Utilisation d'extraits végétaux pour la maîtrise du risque mycotoxique dans les systèmes agro-alimentaires

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Résumé

Les mycotoxines sont des métabolites secondaires toxiques sécrétés par des champignons filamenteux, appartenant essentiellement aux genres *Aspergillus, Fusarium* et *Penicillium,* dans les productions alimentaires d'origine végétale. La contamination par les moisissures toxinogènes entraîne des pertes économiques importantes. Ces molécules sont en outre résistantes aux traitements de transformation comme la cuisson ou la torréfaction et elles peuvent donc se retrouver dans toute la chaîne alimentaire. Une trentaine d'entre elles sont reconnues pour leurs effets néfastes pour la santé humaine et animale. L'utilisation de fongicides est actuellement le moyen le plus utilisé pour lutter contre le risque mycotoxique. Toutefois, dans un contexte de développement durable, des alternatives basées sur le biocontrôle sont à développer. Le travail réalisé a donc eu pour objectif d'étudier in vitro un moyen de lutte biologique pouvant réduire la contamination par des mycotoxines considérées comme préoccupantes et produites par certaines souches espèces fongiques, *Aspergillus flavus*, *Aspergillus carbonarius* et *Fusarium verticillioides,* respectivement productrices d'aflatoxine B1, ochratoxine A et fumonisine B1. Les extraits de plantes sont connus depuis longtemps comme agents antifongiques et la première stratégie a été d'utiliser un produit commercial de lutte biologique, l'Antoferine®, qui est un extrait naturel de vigne contenant des polyphénols, produit par la société Antofénol (Plestan, France). Ce produit a déjà été testé pour son activité antifongique mais c'est la première étude consacrée à son impact sur les mycotoxines. La seconde stratégie a été de tester in vitro trois huiles essentielles extraites de *Cymbopogon schoenanthus, Cymbopogon nardus* et *Eucalyptus camaldulensis*. L'Antoferine® a montré une activité dose-dépendante sur la croissance fongique et la production de toxines, et dans une moindre mesure sur la production de spores, d'*A. flavus* et de *F. verticillioides.* Son effet sur la croissance d'*A. carbonarius* a été plus contrasté puisque la croissance n'était que faiblement impactée et la production de toxines stimulée. L'Antoferine® a également montré un potentiel de détoxification de l'aflatoxine B1 produite par *A. flavus*. Concernant les trois huiles essentielles, des effets inhibiteurs ont été mis en évidence sur la croissance et la production de mycotoxines d'*A. flavus*, *A. carbonarius* et *F. verticillioides* en utilisant une méthode de contact direct et une méthode de diffusion sur disque. D'une manière générale, les huiles essentielles de *Cymbopogon schoenanthus* et de *Cymbopogon nardus* ont montré une activité supérieure à celle de l'huile essentielle d'*Eucalyptus camaldulensis* mais il est intéressant de noter que *E. camaldulensis* a été capable d'inhiber la production de mycotoxines sans effet significatif ou moindre sur le développement fongique.

Abstract

Mycotoxins are toxic secondary metabolites secreted by filamentous fungi, mainly belonging to the genera *Aspergillus, Fusarium* and *Penicillium*, in food productions of plant origin. Contamination by toxigenic molds causes significant economic losses. These molecules are also resistant to processing treatments such as cooking or roasting and can therefore be found in the entire food chain. About thirty of them are recognized for their harmful effects on human and animal health. The use of fungicides is currently the most used means to fight against the mycotoxic risk. However, in a context of sustainable development, alternatives based on biocontrol are to be developed. The objective of this work was to study a biological control method that could reduce the contamination by mycotoxins considered to be of concern and produced by certain fungal strains, *Aspergillus flavus, Aspergillus carbonarius* and *Fusarium verticillioides*, which produce aflatoxin B1, ochratoxin A and fumonisin B1 respectively. Plant extracts have long been known as antifungal agents and the first strategy was to use a commercial biological control product, Antoferine®, which is a natural vine extract containing polyphenols, produced by the company Antofénol (Plestan, France). This product has already been tested for its antifungal activity but this is the first study devoted to its impact on mycotoxins. The second strategy was to test three essential oils extracted from *Cymbopogon schoenanthus, Cymbopogon nardus* and *Eucalyptus camaldulensis*. Antoferine® showed a dose-dependent activity on fungal growth and toxin production, and to a lesser extent on spore production, of *A. flavus* and *F. verticillioides*. Its effect on the growth of *A. carbonarius* was more contrasted since growth was only slightly impacted and toxin production was stimulated. Antoferine® also showed a potential detoxification of aflatoxin B1 produced by *A. flavus*. Concerning the three essential oils, inhibitory effects were demonstrated on the growth and mycotoxin production of *A. flavus, A. carbonarius* and *F. verticillioides* using a direct contact method and a disk diffusion method. In general, the essential oils of *Cymbopogon schoenanthus* and *Cymbopogon nardus* showed higher activity than *Eucalyptus camaldulensis* essential oil but it is interesting to note that *E. camaldulensis* was able to inhibit mycotoxin production with no or lesser effect on fungal growth.

Valorisation des travaux de recherche

Publications

- Chelaghema A., Strub C., Colas de la Noue A., Schorr-Galindo S., & Fontana A. (2021). **Plants for plants: Would the solution against mycotoxins be the use of plant extracts?** In "Mycotoxins in Food and Beverage: Innovations and Advance - Part II". Montet D., Brabet C., Galindo S. and Ray R. C. (Eds), Food Biology series, CRC Press, USA, pp 154-174.
- Chelaghema A., Durand N., Jourdan C., Schorr-Galindo S., Strub C., & Fontana A. **"Antifungal activity of Antoferine®, a commercial biocontrol product against three mycotoxinogenic fungi".** Soumis (Juillet 2021) dans le journal Mycotoxin Research.
- Chelaghema A., Durand N., Servent A., Mamouni M., Poucheret P., Schorr-Galindo S., Fontana A., & Strub C. **"Antifungal and antimycotoxic activities of 3 essential oils against 3 mycotoxinogenic fungi".** Soumis (Octobre 2021) dans le journal Archives of Microbiology.

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- Chelaghema A., Strub C., Schorr-Galindo S., Durand N., Poucheret P., Michel A., & Fontana A. 2020. Utilisation des huiles essentielles pour la maîtrise du risque mycotoxine en agro-alimentaire. 8ème édition des Journées Mycotoxines. Brest (France), 30-31 janvier. (Poster et short présentation).
- Chelaghema A., Strub C., Schorr-Galindo S., Durand N., Poucheret P., Michel A., & Fontana A., 2020. Use of essential oils for the control of mycotoxin risk in agro-food systems. Third International Symposium: Medicinal Plants and Materials (MPM-2020), Tebessa (Algérie), 25 au 27 février. (Communication oral)
- Chelaghema A., Strub C., Schorr-Galindo S., Durand N., Poucheret P., Michel A., & Fontana A. 2019. Use of plant extracts for mycotoxin risk control in agro-food systems. Journée APAB (13 Juin 2019) (poster et short présentation).

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Liste des abréviations

ADN : Acide Désoxyribonucléique

ADNc : ADN complémentaire

AFB1 : Aflatoxine B1

ARN : Acide ribonucléique

CPG/SM : Chromatographie en Phase Gazeuse couplé à un Spectromètre de Masse

FB1 : Fumonisine B1

HPLC : Chromatographie en phase liquide haute performance

HPLC-MS-MS : Chromatographie en phase liquide haute performance-spectrométrie de masse

GC-MS : Chromatographie en phase gazeuse - Spectrométrie de masse

PBS : Tampon phosphate saline

PDA : Potato Dextrose Agar

qPCR : Quantitative Polymerase Chain Reaction

RPM : Rotation par minute

RT : Reverse Transcription

OTA : Ochratoxine A

Introduction Générale

Malgré les progrès de la science et de la technologie, les contaminations par des souches fongiques phytopathogènes et/ou toxinogènes restent parmi les principaux problèmes de la production alimentaire d'origine végétale (Gemeda et al., 2014). Ces pathogènes fongiques sont reconnus comme des pathogènes de terrain et des micro-organismes saprophytes de stockage, produisant des métabolites secondaires, dont les mycotoxines (Sarmast et al., 2021). Près de 25% des denrées alimentaires sont éliminées chaque année en raison de leur contamination par les mycotoxines (Dwivedy et al., 2016; Eskola et al., 2020).

Les effets toxiques des mycotoxines ont été découverts pour la première fois après l'apparition de la maladie de Turkey X au Royaume-Uni dans les années 1960, touchant des dindons nourris avec de la farine d'arachides contaminée par les aflatoxines produites par des souches toxinogènes (*Aspergillus flavus*) (Blount, 1960). Depuis cette période, le répertoire des mycotoxines découvertes ne cesse de s'agrandir.

De nos jours, plus de 400 mycotoxines sont connues, et la plupart d'entre elles sont produites par des espèces appartenant à l'un des trois genres de champignons suivants : *Aspergillus, Penicillium* et *Fusarium*. Plusieurs mycotoxines différentes ont été identifiées pour leurs effets sur la santé humaine et animale : l'ochratoxine A, la zéaralénone, les aflatoxines, les trichothécènes, la patuline, les fumonisines et la citrinine (Ashiq et al., 2014 ;Tao et al., 2018; Haque et al., 2020).

Cependant, le premier rôle des métabolites secondaires des champignons est d'ordre écologique. Ainsi, certains de ces métabolites secondaires agissent comme des facteurs de virulence chez les champignons pathogènes des animaux et des végétaux, alors que d'autres sont à même de protéger leur hôte de stress biotiques ou abiotiques. La présence des mycotoxines est plus importantes dans les environnements chauds et humides qui favorisent la croissance des champignons et la production de mycotoxines (Soares et al., 2018).

Ces composés toxiques sont relativement stables lors des traitements thermiques utilisés dans la préparation de divers produits (Wambacq et al., 2016). La consommation de produits contaminées par des mycotoxines est à l'origine de problèmes pour la santé humaine et animale, de l'augmentation des coûts de santé et des soins vétérinaires, de la diminution de l'efficacité de la production ou de l'impossibilité de commercialiser les produits dans le pays ou à l'étranger.

Diverses organisations nationales et internationales de santé publique, comme le Centre international de recherche sur le cancer (CIRC), l'Organisation mondiale de la santé (OMS), la

FAO et l'Union européenne (UE) ont établi certaines limites maximales pour les mycotoxines dans les aliments pour l'importation et l'exportation des produits concernés (Haque et al., 2020). Pour toutes ces raisons, il semble essentiel de trouver des moyens de lutte contre la production des mycotoxines ainsi que des moyens de détoxification des produits agricoles contaminés.

L'emploi des fongicides fait partie des solutions les plus couramment utilisées pour la lutte contre les champignons mycotoxinogènes. Mais certains de ces composés posent différents problèmes environnementaux et de santé humaine, en particulier pour les agriculteurs qui les manipulent à haute concentration. Parmi ces fongicides chimiques, l'imazalil, le thiabendazole et le bromure de méthyle sont couramment utilisés. Des efforts ont été accomplis pour découvrir et développer de nouveaux produits plus écologiques connus sous le nom de biofongicides, organismes vivants ou produits issus de ces organismes ayant la particularité de limiter ou de supprimer les ennemis des cultures (Thakore, 2006). Ces produits sont moins toxiques que leurs homologues chimiques. Cette découverte a permis à la fois de réduire l'utilisation excessive des fongicides chimiques en agriculture et de diminuer leur impact néfaste sur l'environnement et la santé humaine. Parmi les méthodes alternatives récemment explorées, l'utilisation d'extraits des plantes se présente comme une voie d'intérêt.

Tous les êtres vivants ont un métabolisme primaire qui fournit les molécules de base (acides nucléiques, lipides, protéines, acides aminés et glucides). Les plantes produisent, en plus, un grand nombre de composés qui ne sont pas issus directement de la photosynthèse, mais résultent des réactions chimiques ultérieures. Ces composés sont appelés métabolites secondaires. Ce sont des molécules ayant une répartition limitée dans l'organisme de la plante. Ils y jouent différents rôles, dont celui de la défense contre les agressions externes. Ces métabolites ne sont pas toujours nécessaires à la survie de la plante. Les plantes aromatiques et médicinales jouent un rôle économique considérable dans les secteurs de la parfumerie, des cosmétiques, de la pharmacie et des industries de l'agroalimentaire. En effet, les plantes représentent une source durable de remèdes traditionnels et efficaces grâce aux principes actifs qu'elles contiennent : alcaloïdes, flavonoïdes, vitamines……et huiles essentielles.

De nos jours, les huiles essentielles suscitent de plus en plus l'intérêt des chimistes, médecins, biologistes et industriels de l'agroalimentaire en raison de leurs utilisations dans le traitement de certaines maladies infectieuses pour lesquelles les antibiotiques de synthèse deviennent de moins en moins actifs ou dans la préservation des aliments contre l'oxydation comme alternatives aux produits chimiques de synthèse.

C’est dans ce contexte que ce travail de thèse a été consacré à l’étude des effets antifongiques et/ou antimycotoxiques de trois huiles essentielles issues de trois plantes (*Cymbopogon nardus et Cymbopogon schoenanthus* appartient à la famille de Poaceae, *Eucalyptus camaldulensis* appartient à ma famille de *Myrtaceae* ainsi que d’un produit commercial issu des déchets de la vigne, l’Antoferine®.

Le travail présenté dans ce manuscrit est réparti en trois chapitres :

Le premier chapitre est une synthèse bibliographique concernant (i) les principaux champignons filamenteux mycotoxinogènes et leurs mycotoxines ainsi que les extraits végétaux utilisés pour leur biocontrôle, (ii) l’Antoferine® et (iii) les huiles essentielles utilisées dans cette étude.

Le deuxième chapitre expose les résultats obtenus au cours de ce travail de thèse, sous la forme de deux articles, l’un consacré à l’Antoferine® et le second, aux huiles essentielles. Le troisième chapitre présente une conclusion générale et des perspectives de travaux.

Dans notre étude, nous nous sommes attachés à étudier les effets de l’Antoferine® et les trois huiles essentielles citées précédemment sur (1) la croissance de trois souches mycotoxinogènes : *A. flavus*, *A. carbonarius* et *F. verticillioides* (2) la sporulation des souches (3) leurs capacités à produire l’aflatoxine B1, l’ochratoxine A et la fumonisine B1, respectivement, quand ce champignon est soumis à de différentes conditions de culture (différentes concentrations de ces extraits).

Afin de mieux comprendre l’effet de ces extraits, il est indispensable de comprendre leur mécanisme d’action au niveau moléculaire. De ce fait, nous nous sommes focalisés sur l’étude de la variation dans l’expression de gènes de biosynthèse de l’AFB1 et FB1 chez *A. flavus* et *F. verticillioides* mis en culture en présence de l’Antoferine® et des différentes huiles essentielles afin de pouvoir corréler ces variations aux niveaux de production de ces deux mycotoxines.

Nous avons également testé l’effet de l’Antoferine® sur la détoxification de l’AFB1 et de la FB1. Enfin nous avons réalisé des études en microscopies électroniques à balayage et à transmission afin d’observer l’effet de l’Antoferine® sur l’ultrastructure d’*A. flavus* qui produit l'AFB1, classé comme cancérigène par le IARC (Centre International de Recherche sur le Cancer).

Synthèse Bibliographique

1 Synthèse bibliographique

Cette synthèse bibliographique est organisée en trois parties. La première correspond à une revue bibliographique publiée sous forme de chapitre d'ouvrage (Chelaghema A., Strub C., Colas de la Noue A., Schorr-Galindo S., & Fontana A. (2021). Plants for plants: Would the solution against mycotoxins be the use of plant extracts? In "Mycotoxins in Food and Beverage: Innovations and Advance - Part II". Montet D., Brabet C., Galindo S. and Ray R. C. (Eds), Food Biology series, CRC Press, USA, pp 154-174.) dont le but était de décrire les principaux champignons filamenteux mycotoxinogènes et leurs mycotoxines, les extraits de plantes utilisés pour le biocontrôle de la contamination par les mycotoxines ainsi que les mécanismes d'action impliqués dans l'inhibition de la croissance fongique et/ou de la contamination par les mycotoxines par les extraits de plantes. La seconde partie présente l'Antoferine®, un éco-extrait polyphénolique de la vigne, produit par la société Antofénol et dont les propriétés antifongiques et antimycotoxiques ont été étudiées au cours de ce travail de thèse. La dernière partie est consacrée aux huiles essentielles et plus spécifiquement aux huiles essentielles de *Cymbopogon nardus, Cymbopogon schoenanthus* et *Eucalyptus camaldulensis* qui ont également été testées pour leur activités sur la croissance fongique et la mycotoxinogenèse.

1.1 Des plantes pour les plantes : La solution contre les mycotoxines serait-elle l'utilisation d'extraits de plantes ? Plants for plants: Would the solution against mycotoxins be the use of plant extracts?

CHAPTER
6

Plants for Plants: Would the Solution Against Mycotoxins be the Use of Plant Extracts?

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1. Introduction

Many food commodities are contaminated by filamentous fungi and the toxins produced by some of them, travelling from the field to the plate. These contaminations cause important health risks to humans and animals, and produce losses whether in quality or quantity or both (Hu et al. 2017). The Food and Agriculture Organisation (FAO) estimates that around 1000 million tons of food are damaged each year by mycotoxigenic filamentous fungi (Prakash et al. 2015). For years the only solution used against moulds was chemical treatments, but unfortunately some of them have adverse effects on the health of human and animals (Kiran et al. 2016), and their use could be limited because of their inconveniences such as the resistance of fungi to fungicides, the toxicity of residual fungicides and the pollution of the environment. As a result, alternative strategies can be explored to reduce the occurrence of mycotoxins in food, based on the use of biological methods that do not affect human health or the environment. This strategy consists of the use of physical methods, biocontrol agents and herbal extracts (Mari et al. 2016, Divband et al. 2017). To this end, the use of natural compounds possessing antifungal activities seems interesting, due to their low non-toxic effects for the environment, and their effect on animal and human health (Nicosia et al. 2016). When attacked by pathogens or as part of their metabolism, plants produce a wide variety of compounds with a large diversity in their activities. Among all these molecules, some are particularly efficient at reducing the growth of fungi and their toxins (Divband et al. 2017).

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In this context, the aim of this chapter is to describe: (1) the main mycotoxigenic filamentous fungi and their mycotoxins, (2) the plant extracts used for the biocontrol of mycotoxin contamination, (3) the mechanismsof action involved in the inhibition of fungal growth and/or mycotoxin contamination by plant extracts.

2. Main Mycotoxinogenic Species and Their Regulated Mycotoxins

2.1 *Fusarium* and Fusariotoxins

Fusarium species widely contaminates grain products and are one of the most dangerous risks in crop productions (Da Silva Bomfim et al. 2015). The contamination with *Fusarium* species is usually linked to infection and accumulation of harmful mycotoxins, such as trichothecenes (T-2, HT-2, deoxynivalenol and nivalenol), zearalenone and fumonisins. Some species can produce several mycotoxins simultaneously like *Fusarium graminearum*, which is considered to be the most virulent among the *Fusarium* species producing trichothecenes. *F. graminearum* can also produce the zearalenone and some other secondary metabolites such as culmorin, sambucinol, dihydroxy-calconectrin, butenolide and fusarin C which are more or less toxic (Nguyen et al. 2017). Deoxynivalenol is the most reported *Fusarium* toxin in the world (Arraché et al. 2018) and it is the only member of trichothecenes under regulation by the European Commission (European Commission 2006). The main *Fusarium* species involved in zearalenone production is *Fusarium graminearum* but other members such as *Fusarium roseum, Fusarium culmorum, Fusarium oxysporum* and *Fusarium equiseti* are also implied (Yang et al. 2018). Zearalenone is highly prevalent in various crops, particularly maize, but also in barley, wheat, oats, rice and sorghum and is regulated by the European Commission (European Commission 2006). Fumonisins are mainly produced by *Fusarium verticillioides*. Fumonisins have been classified into four groups within which group B is of the most concern. It includes fumonisins B1, B2 and B3. Fumonisin B1represents the greatest health risk due to its high toxicity and is responsible for 70 to 80% of the fumonisin contamination while fumonisin B2 accounts for 15 to 25% and fumonisin B3 is poorly involved with 3 to 8% of contaminated foodstuffs (Da Silva Bomfim et al. 2015). They are classified in group 2B by the International Agency for Research on Cancer because of their potential carcinogenic effect on humans (Ostry et al. 2017). Thus, maximum levels for fumonisins have been established by the European Commission (Shephard et al. 2019).

2.2 *Aspergillus* and Associated Mycotoxins

The genus *Aspergillus* is dominant among food deteriorating moulds. *Aspergillus* species widely occur in foodstuffs, particularly in starchy cereal grains such as wheat, maize, sorghum, rice, barley and millet where they may produce toxic secondary metabolites like aflatoxins and ochratoxin A (Sultana et al. 2015).

Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* which are the main contaminant of peanuts, maize, pistachio and other crops in tropical and subtropical areas, leading to important economic losses. Aflatoxins are divided in four major groups namely B1, B2, G1 and G2 with aflatoxin B1 being the most toxic and classified in Group 1, i.e. carcinogenic to humans, by the International Agency for Research on Cancer (Ibrahim et al. 2017, Ostry et al. 2017). The European Commission set maximum limits for aflatoxinB1 and total aflatoxins (European Commission 2006). Ochratoxin A is usually produced by *Aspergillus* and *Penicillium* species. This toxin can be found in numerous crops, foods and beverages such as cereals, pulses, coffee, beer, wine and grape juice as well as dried vine fruits, nuts, cacao products and spices (El Khoury et al. 2017a). Ochratoxin A has been classified in Group 2B, i.e. possibly carcinogenic to humans, by the International Agency for Research on Cancer (Ostry et al. 2017). The European Union has set regulations for ochratoxin A in many food products (European Commission 2006).

2.3 *Penicillium* and Associated Mycotoxins

The main species of the genus *Penicillium* are *P. chrysogenum, P. expansum, P. verrucosum, P. viridicatum, P. oxalcum, P. brevicompactum, P. cyclopium, P. roqueforti* and *P. velutinum*. They can be present in cereal grains from the field to their storage, as well as in cereal-derived products (Nguyen et al. 2017). Strains of the *Penicillium* genus can produce mycotoxins such as patulin, citrinin and ochratoxin A with potential damage to human health (Cruz et al. 2016).Some of them produce only one mycotoxin while other species can produce several toxins simultaneously. *Penicillium expansum* is known to be the most important producer of patulin (Li et al. 2019). Patulin is found in fruits, fruit products, vegetables, cereals, and cheese. Patulin has become a major concern with regard to public health (Diao et al. 2018). *Penicillium verrucosum* is the main ochratoxin A producer among *Penicillium* sp. (Pittand Hocking 2009). Citrinin is a mycotoxin produced by several species of the genera *Aspergillus* and *Penicillium*, and its presence is generally concomitant with ochratoxin A in food and feed (Degen et al. 2018). Citrinin was firstly isolated from a culture of *Penicillium citrinum* and has been further associated with other species (e.g. *A. ochraceus, P. verrucosum*). Citrinin and ochratoxin A often co-occurs in cereals and plant products. Indeed, some species such as *P. verrucosum* are producing both mycotoxins concomitantly.

3. Plant Extracts Used for the Biocontrol of Mycotoxin Contamination

During the last 25 years, many *in vitro* and *in vivo* investigations have been carried out for the development of new plant-based formulations for their use in agriculture pest management programs. One of the main pros of botanical products resides in their biodegradability which make them good candidates for eco-chemical products and for their use as a bio-rational approach in

integrated plant protection programs (Prakash et al. 2015). Into the higher plant reign, a large set of secondary metabolites (e.g. phenolic compounds, tannins, alkaloids etc.) are produced to protect themselves against various kind of aggressions (mechanical, biological or climatic). Many studies have been conducted in the last few years and essential oils, aqueous or alcoholic plant extracts have progressively shown their efficiency against fungal growth and/or mycotoxins production (Ramirez-Mares and Hernandez-Carlos 2015, Moon et al. 2018, Abdullah et al. 2019, Chen et al. 2019).

These researches are summarized in Table 1 and the most recent are detailed below. *Aspergillus* and *Fusarium* are the fungal genera most commonly used to test essential oils and plant extracts, followed by *Penicillium* (Kumar et al. 2007). The production of aflatoxin B1 by *A. flavus* was inhibited using piperine, a main component of black and long peppers (*Piper nigrum* L. and *Piper longum* L.) by 30% at 0.0006 mM of piperine (lyophilized piperine standard, purity ≥97.0%) while no detectable levels of toxin were observed using 0.17 mM of piperine. At the same time, growth inhibition was only of 35% with 0.17 mM of piperine. Using 0.04 mM of piperine allowed an aflatoxin B1 inhibition of 95% with only a growth inhibition of 12% (Caceres et al. 2017). *Cinnamomum zeylanicum* Blume essential oil exhibited high toxicity against all the tested fungi (*Aspergillus flavus, A. niger, A. candidus, A. sydowi, A. fumigatus, Cladosporium cladosporoides, Curvularialunata, Alternaria alternata, Penicillium* sp. and *Mucor* sp.) including an high toxigenic species of *A. flavus* and its minimum inhibitory concentration (MIC) ranged between 0.25 and 6.0 µL/mL culture medium. In addition, *Cinnamomum zeylanicum* Blume essential oil completely inhibited at 0.3 µL/mL the production of aflatoxin B1 (Kiran et al. 2016). Matusinsky et al. (2015) examined essential oils extracted from *Pimpinella anisum, Thymus vulgaris, Pelargonium odoratissimum, Rosmarinus officinalis* and *Foeniculum vulgare* against the fungi *Oculimaculay allundae, Microdochium nivale, Zymoseptoria tritici, Pyrenophora teres* and *Fusarium culmorum.* These essential oils affected the growth of the fungal isolates differently. Indeed, essential oils of *T. vulgaris* and *P. odoratissimum* inhibited the growth of targeted fungal species. *P. odoratissimum* essential oil inhibited the growth of *O. yallundae, P.teres* and *Z. tritici*at 1 µL/mL while *F. culmorum* and *M. nivale* growths were inhibited at 5 µL/mL. Sharma et al. (2017) reported that essential oils obtained from clove (*Syzygium aromaticum*), lemongrass (*Cymbopogon citratus*), mint *(Mentha piperita*) and eucalyptus (*Eucalyptus globulus*) were effective against the wilt causing fungus *Fusarium oxysporum* f.sp. *lycopersici* 1322. The inhibitory effect of oils showed a dose-dependent activity on the tested fungus. Complete inhibition of mycelial growth and spore germination was observed using clove oil at 125 ppm with IC50 value of 18.2 and 0.3 ppm, respectively. Phytochemicals (methyl-trans-p-coumarate, methyl caffeate, syringic acid and ursolic acid) at different concentrations (500, 750 and 1000 ppm) were tested against *Alternaria alternata, Curvularia lunata, Fusarium moniliforme, F. pallidoroseum* and *Helmintho sporium* sp. All tested phytochemicals affected the growth inhibition of all fungi except *A. alternata*, which was found to be resistant to syringic acid (Shaik et al. 2016). Pane et al. (2016) showed an

Table 1. Efficacy of plant extracts against fungal growth and mycotoxin production

Plants	Target	Remarks	References
Myrcia splendens (dichloromethane extract of mature leaves)	*Alternaria alternata*	Inhibitory effect (10.2%) on the mycelial growth at 1 mg/mL.	Pontes et al. (2019)
Micromeria graeca (aqueous extract)	*Aspergillus flavus*	Inhibitory effect on the production of aflatoxin B1 for *Aspergillus flavus* strain E28 (77.7%), strain E73 (70.8%) and strain NRRL 62477 (99.2%) at the dose of 10 mg/mL. At 10 mg/mL, aflatoxin inhibition was accompanied by a mild increase of the colony diameter.	El Khoury et al. (2017)
Curcuma longa L. (turmeric essential oil)	*Aspergillus flavus*	The essential oil decreased the fungal mycelia growth (93.4%) at the concentration of 8 µL/mL The aflatoxinB1 inhibition rate of 8 µL/mL turmeric essential oil was 78.4%.	Hu et al. (2017)
Thymus vulgaris L. (essential oil) *Mentha x piperita* L. (essential oil) *Rosmarinus officinalis* L. (essential oil) *Lavandula angustifolia* Mill. (essential oil)	*Fusarium verticillioides*	100% inhibition of growth at the concentration of 250, 500 and 1000 µL/L after 12 days of incubation. 34.1% inhibition of growth at the concentration of 1000 µL/L after 12 days of incubation. 8.2% inhibition of growth at the concentration of 1000 µL/L after 12 days of incubation. 21.8% inhibition of growth at the concentration of 1000 µL/L after 12 days of incubation.	Vilaplana et al. (2018)
Bay leaves, cumin, fenugreek, melissa, mint and sage (essential oils and phenolic compounds) Anise, chamomile, fennel, rosemary and thyme (phenolic compounds)	*Aspergillus carbonarius*	Inhibitory effect on the production of ochratoxin A between 25% (sage) and 80% (melissa) for all essential oils (5 µL/mL) and between 13% (thyme) and 69% (mint) for phenolic compounds (250 µg GAE/mL). Essential oils had a greater impact than the phenolic extracts on the ochratoxin A production.	El Khoury et al. (2016)

Chamaemelum nobile *Cuminum cyminum* *Zingiber officinale* *Ziziphora clinopodioides,* *Thymus vulgaris* (essential oils)	*A. niger* *A. fumigatus* *A. flavus* *A. ochraceus* *P. citrinum* *P. chrysogenum*	Strong activity (expressed in MIC - minimum inhibitory concentrations - mean values) against tested fungal strains for the oil of *T. vulgaris* (1250 µg/mL), followed by *Cu. Cyminum* (1416 µg /mL), *Zin. officinale* (1833 µg/mL), *Ziz. clinopodioides* (2166 µg/mL) and *Ch. nobile* (3750 µg/mL).	Sharifzadeh et al. (2016)
Rosmarinus officinal (essential oil)	*Aspergillus niger*	From the concentration of 0.50%, a complete absence of growth was observed for the 16 tested isolates.	Baghloul et al. (2016)
Cinnamomum zeylanicum Blume (essential oil)	*A. flavus* *A. niger*	The CZ essential oil caused complete inhibition of all test molds and aflatoxin B1 secretion at 0.25 to 0.6 µg/mL and 0.3 µg/mL respectively.	Kiran et al. (2016)
Parastrephia quadrangularis Extract A: Aerial parts with capitulum in ripe fruits. Extract B: Aerial parts with capitulum in flowering	*Fusarium verticillioides*	*P. quadrangularis* extract A had a significant activity on the growth of *F. verticillioides* M7075 with an MIC of 118.74 µg/mL whereas MIC of extract B was 250 µg/mL.	Abdullah et al. (2017)
Residues from essential oil industry: *Hyssopus officinalis* L., *Thymus mastichina* L., *Lavandula × intermedia* var. Super and *Salvia lavandulifolia* Vahl. (ethanolic extracts)	*Penicillium verrucosum*	The most active extracts were those from the solid residues of *Hyssopus officinalis* with 50% inhibition of fungal growth for 92 mg/mL (EC_{50} – median effective concentration)	De Elguea-Culebras et al. (2016)
Piper betle L. var. Tamluk Mitha (essential oil)	*Penicillium expansum*	Inhibition zones of 42.8±1 mm and 53.8±2 mm were observed for 10 and 30 µL of essential oil, respectively, using disc diffusion method and of 28.7±1 mm and 38.5±1 mm for 10 and 30 µL of essential oil, respectively, using disc volatilization method.	Basak and Guha (2015)

(*Contd.*)

Table 1. (*Contd.*)

Plants	*Target*	*Remarks*	*References*
Thymus vulgaris L. (essential oil)	*Aspergillus flavus*	MIC and MFC (minimum fungicidal concentration) were found at 250 µg/mL. Inhibition of the production of both B1 and B2 aflatoxins was observed at 150 µg/mL.	Kohiyama et al. (2015)
Rosmarinus officinalis L. (essential oil)	*Fusarium verticillioides*	The mycelial growth of *Fusarium verticillioides* (Sacc.) Nirenberg was reduced significantly from 150 µg/mL and the production of fumonisins B1 and B2 from the concentration of 300 µg/mL.	Da Silva Bomfim et al. (2015)
Lippia scaberrima, Lippia rehmannii Mentha spicata, Helichrysum splendidum, Cymbopogon citratus, Thymus vulgaris, Syzygium aromaticum, Cinnamon zeylanicum and Cinnamomum camphora (essential oils) R-(−)-carvone, S-(+)-carvone, eugenol, limonene, neral, citral and thymol) (pure compounds)	*Fusarium oxysporum*	Inhibition of growth of *F. oxysporum* S-1187 was obtained from 500 µL/L with *Thymus vulgaris, Syzygiumaro maticum* essential oil, as well as pure citral, eugenol and thymol.	Manganyi et al. (2015)
Punicagranatum L. (peel methanol extract)	*F. sambucinum*	MIC and MFC were found to be 20 and 120 mg/mL, respectively. 75.5% inhibition on mycelial growth and complete inhibition on spore germination was observed at the concentration of 20 mg/mL.	Elsherbiny et al. (2016)
Punica granatum L. (peel concentrated aqueous extract)	*Penicillium digitatum Penicillium expansum Botrytis cinerea*	Inhibition of conidia germination was significantly inhibited by 100, 91.0 and 82.7% for *B. cinerea, P. digitatum* and *P. expansum*, respectively, using 12 g/L extract. Viability of conidia was 0%, 20% and 42.3% for *B. cinerea, P. digitatum* and *P. expansum*, respectively, using 12 g/L extract.	Nicosia et al. (2016)

Parastrephia lepidophylla (ethanolic extract)	*Penicillium digitatum*	MIC and MFC were found to be 150 mg of GAE/mL and 350 mg GAE/mL. Conidia germination was inhibited from 200 mg GAE/mL.	Ruiz et al. (2016)
Basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme (essential oils)	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Penicillium chrysogenum.*	Oregano, thyme and cinnamon were the best effective essential oil considering MIC. Combination of oregano and thyme showed in a synergistic effect against *A. flavus*, *A. parasiticus* and *P. chrysogenum* while association of peppermint and tea tree resulted in a synergistic effect against *A. niger*	Hossain et al. (2016)
Curcuma longa (ethanol extract) and its major compounds (curdione, isocurcumenol, curcumenol, curzerene, β-elemene, curcumin, germacrone and curcumol)	*Fusarium graminearum*	*Curcuma longa* had a strong inhibitory effect on *Fusarium graminarium.* Eight chemical constituents of *Curcuma longa* had inhibitory effects on the mycelia growth of *F. graminearum*. Among the eight chemicals, curdione had the best inhibitory effect on the growth of *F. graminearum* with an inhibitory rate of 52.9%.	Chen et al. (2018)
Borreria verticillata, *Lactuca capens*is, *Colocasiae sculenta*, *Jatropha gossy* and *Ageratum conyzoide* extracts (ethanolic, hot and cold-water extracts)	*Aspergillus flavus*, *A. parasiticus* and *A. ochraceus*	The extracts of *Borreria verticillata* inhibited the growth of the tested fungal strains. *Borreria verticillata* appeared to be more effective as an antifungal agent than the other plant extracts. The ethanolic extracts were more effective than the cold and hot water extracts.	Jeff-Agboola and Onifade (2016)

dose-dependent inhibition of the mycelial growth and conidia germination of *Alternaria alternate* using crude foliar extracts of a wild *Capsicum annuum* accession. Furthermore, a reduced soft rot disease severity was observed on artificially infected ripe cherry tomatoes treated with these extracts.The methanol extract of pomegranate peels at the concentration of 20 mg/mL was tested against *Fusarium sambucinum.* The results showed that mycelial growth of *F. sambucinum* was inhibited at 75.5%, while spore germination was completely inhibited. The extract exhibited a minimum inhibitory concentration (MIC) of 20 mg/mL and a minimum fungicidal concentration (MFC) of 120 mg/mL (Elsherbiny et al. 2016). Chen et al. (2018) observed that *Curcuma longa* alcohol extract had a strong inhibitory effect on various pathogenic fungi including *Fusarium graminearum, Fusarium chlamydosporum, Alternaria alternata, Fusarium tricinctum, Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium culmorum, Rhizopusoryzae, Cladosporium cladosporioides, Fusarium oxysporum* and *Colletotrichum higginsianum*. Another study by Jeff-Agboola and Onifade (2016) showed that the extracts of *Borreria verticillata, Lactuca capenisi, Colocasiaes culenta* and *Jatropha gossy* exhibited antifungal activity against *A. flavus* and *A. parasiticus*, while only the extracts of *Ageratum conyzoide* had inhibitory effect on *A. ochraceus*. An aqueous extract of the medicinal plant *Micromeria graeca* was shown to completely inhibit the aflatoxin production by *Aspergillus flavus* without reducing fungal development. The essential oils of bay leaves, cumin, fenugreek, melissa, mint, and sage showed a great impact on the ochratoxin A production of *Aspergillus carbonarius* on synthetic grape medium at 28°C for four days. Reduction levels ranged between 25% (sage) and 80% (melissa) at 5 µL/mL. Phenolic compounds extracted from bay leaves, cumin, fenugreek, melissa, mint, sage, anise, chamomile, fennel, rosemary and thyme reduced ochratoxin A production and reduction levels ranged between 13% (thyme) and 69% (mint). Although they did not affect the growth of *A. carbonarius* (El Khoury et al. 2017a).

4. Mechanisms of Action Involved in the Inhibition of Fungal Growth and/or Mycotoxin Contamination by Plant Extracts

The action of plant extracts against mycotoxin contamination of food products involves three main mechanisms (anti-fungal and/or anti-mycotoxin synthesis and/or detoxifying activities) that can happen alone or in combination and involve a reduction of mycotoxin levels (Fig. 1).

4.1 Antifungal Activity

The mechanism of action of the plant-based extracts is described less. Their composition rich in bioactive molecules allow for the plant extracts to act simultaneously on several cellular targets. Some of them have been identified: cell membrane, cell wall, mitochondria and efflux pump (Nazzaro et al. 2017).

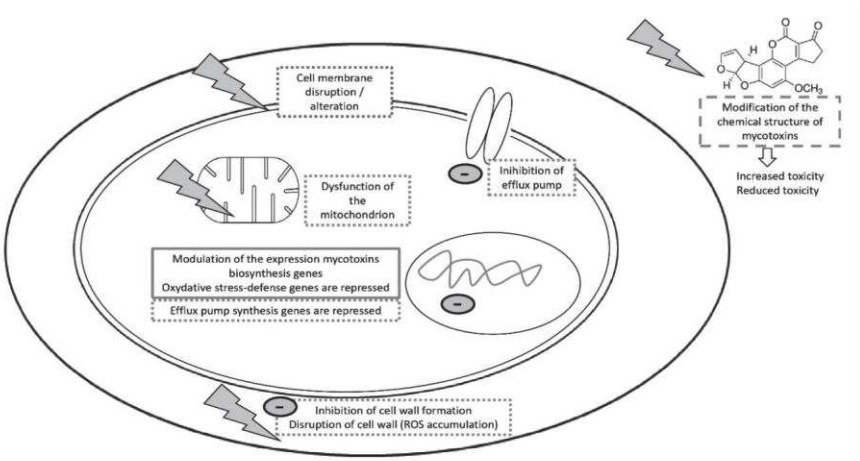

Figure 1. Schematic representation of mechanisms involved in the reduction of mycotoxin contamination (dotted line: fungicide/fungistatic actions, solid line: anti-mycotoxin synthesis activity, dash line: detoxifying activity)

4.1.1 Cell Membrane Disruption, Alteration, and Inhibition of Cell Wall Formation

Plants extracts like essential oils are mixtures of molecules characterized by their poor solubility in water and high hydrophobicity (Jing et al. 2014). This hydrophobicity facilitates their incorporation into plasma membranes and those of intracellular organelles (especially mitochondria). These compounds can then act by altering the lipid membrane composition, like decreasing the ergosterol levels (Kedia et al. 2015). Ergosterol is the main constituent of the fungal cell membrane which is specific and important for fungi and its presence is very important for maintaining the cell function and integrity (Hu et al. 2017). Reduction or absence of ergosterol in fungal membranes leads to a modification in the cell permeability and disruption in cell organelles (Kiran et al. 2016). *Cinnamomum zeylanicum* Blume essential oil in the concentration of 0.5 µg/mL was capable to reduce with a percentage of 100% the ergosterol in the plasma membrane of *Aspergillus flavus*. Such a reduction resulted in a significant leakage of vital cellular ions (Ca^{+2}, K^{+} and Mg^{+2}) in the *Cinnamomum zeylanicum* Blume essential oil fumigated hyphae compared to control. A significant deformation in conidiophores and conspicuous depressions in conidia of *Cinnamomum zeylanicum* Blume essential oil fumigated hyphae compared to control were also observed (Kiran et al. 2016). Essential oil derived from turmeric (*Curcuma longa* L.) was shown to significantly reduce the level of ergosterol of *Aspergillus flavus* (Hu et al. 2017). Inhibition of ergosterol biosynthesis was also detected at a concentration of 100 µg/mL of essential oil of *Thymus vulgaris* L. in *Aspergillus flavus* by Kohiyama et al. (2015). In a research work performed by Da Silva Bomfim et al. (2015), essential oil of *Rosmarinus officinalis* L. at the concentration of 600 µg/mL was capable to inhibit ergosterol production in *Fusarium verticillioides*. Chen et al. (2018) demonstrated that

the utilisation of the extract of *Curcuma longa*, curdione and curcumenol, decreased the ergosterol content in *Fusarium graminearum,* resulting in the inhibition of the growth of the fungal strain. This result is further proof that ergosterol is one of the most important compounds to ensure essential cellular functions, e.g. the integrity of the membrane structure, membrane binding enzyme activity, cell viability and cellular transport. The absence of ergosterol or its decrease cause unnatural function of the fungal cell membrane or yet cell break. Moreover, treatments of hyphae with extracts of *Parastrephia quadrangularis* induced distortion in permeability of plasma membrane in *Fusarium verticillioides* (Abdullah et al. 2017). The fungal cell wall of fungi is mainly composed of different polysaccharides according to taxonomic groups (chitin, glucans, chitosan, chitin, cellulose), with smaller quantities of proteins and glycoproteins (Lagrouh et al. 2017). Chitin and glucans have been used as potential targets to search for new antifungals and many natural products have been identified as inhibitors through stimulating plant defence mechanisms against fungal pathogens which cause destruction of the fungal cell wall (Vicente et al. 2003). Thus, thyme essential oils were capable to induce the activities of chitinase, β-1,3-glucanase in avocado fruit which lead to the degradation of the cell wall of the fungal pathogens (Sivakumar and Bautista-Baños 2014). Anethole, a major component of anise oil, causes a change on the hyphal morphology such as swollen hyphae at the tips and inhibits chitin synthase activity in permeabilized hyphae of *Mucormucedo* IFO 7684 (Yutani et al. 2011). Methanol extract of pomegranate peels inhibited the growth of *Fusarium sambucinum in vitro*. This inhibition was correlated with some morphological modifications in *F. sambucinum* hyphae including curling, twisting and collapse. Alterations of the fungal ultrastructure were also observed including cell empty cavity and the disintegration of cytoplasmic organelles (Elsherbiny et al. 2016).

4.1.2 Dysfunction of the Fungal Mitochondria

Mitochondria are organelles, which are a central part needed to maintain the viability of eukaryotic cells due to their enzyme activities. Mitochondrial enzymes (lactate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, citrate synthetase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase) are key enzymes in respiratory chains of the cells which produce ATP (Siahmoshteh et al. 2018). Some plant extracts can inhibit the action of these enzymes. Hu et al. (2017) have shown that turmeric essential oils could inhibit the activities of mitochondrial ATPase and dehydrogenases. The ATPase inhibitory rate at the concentration of 8 μL/mL of turmeric essential oil was 74.6% in *Aspergillus flavus.* The turmeric essential oil also exhibited inhibition on both succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) activities, which are key enzymes in tricarboxylic acid (TCA) cycle, which is greatly important for energy metabolism of *Aspergillu sflavus.* The citral is a naturally occurring isoprenoid with two isomers (geranial and neral). This compound was found to cause the loss of matrix and increase of irregular mitochondria resulted in the change of the mitochondrial morphology and function of *Penicillium digitatum* (Zheng et al. 2015). The deformation of the mitochondriawas correlated with a decrease in intracellular ATP content and an increase in extracellular ATP content in *Penicillium*

digitatum cells. This result confirmed that citral had a detrimental effect on mitochondrial membrane permeability. Citral was also found to decrease the activities of citrate synthetase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase and increase citric acid content (Zheng et al. 2015). Moreover, inhibitory effect in the tricarboxylic acid cycle (TCA) pathway of *Penicillium digitatum* cells was observed. These results showed that in addition to damaging the permeability of the mitochondrial membrane, citral could disrupt the TCA cycle of *Penicillium digitatum*.

4.1.3 Inhibition of Efflux Pumps

The fungal plasma membrane (PM) H^+-ATPase enzyme plays an important role in eukaryotic cell physiology in maintaining the transmembrane electrochemical proton gradient necessary for nutrient uptake (Kongstad et al. 2015). The fungal PM H^+-ATPase enzyme also allows the export of the toxic substances out of the cell (Lagrouh et al. 2017) and its inhibition could cause the fungal cell death. *Lippiarugosa* essential oil at the concentration of 2000 mg/L completely inhibited the activity of the PM H^+-ATPase in *Aspergillus flavus*. This inhibition was accompanied by an acidification of the external medium (Tatsadjieu et al. 2009).

4.1.4 Reactive Oxygen Species (ROS) Production

Reactive oxygen species (ROS) are natural by-products of cellular metabolism originating from NADPH oxidase located in the plasma membrane. They are also one of the major factors inducing the lipid peroxidation of eukaryotic cell membrane under various environmental stimuli (Gao et al. 2016). Accumulation of intracellular ROS results in oxidative damages affecting important biomolecules such as proteins, DNA and lipids, and can be associated with cell death (Almshawit and Macreadie 2017). Among ROS, NO (nitrous oxide) is a highly active molecule recognized as an intra and inter cellular signalling molecule and its role in fungi remain unclear in literature. The efficiency of dill oil, curcuma longa oil and thymol have been related to an accumulation of ROS in the cytoplasm of fungal cells (OuYang et al. 2018). Shen et al. (2016) showed that thymol provoke the lysis of *Aspergillus flavus* spore via the induction of nitric oxide. OuYang et al. (2018) also demonstrated that citral increased the accumulation of ROS in *Penicillium digitatum.* As well, *Rosmarinus officinalis* L. essential oil inhibits fumonisin production by *Fusarium verticillioides* as a consequence of cell wall perturbation by ROS leading to the loss of cellular components (Da Silva Bomfim et al. 2015).

4.2 Mycotoxin Synthesis Inhibition Using Plants Extracts

The frequency of contamination of global crops by mycotoxins shows that the strategies currently used are insufficient to ensure the safety of food and that it is necessary to develop others, in addition to, or replacing existing ones. In this context, strategies based on the use of natural compounds, generally recognized as not harmful to the environment and to health,

seem interesting. Caceres et al. (2017) demonstrated that piperine inhibited aflatoxin production correlated with an enhancement of antioxidant status in *A. flavus*. This inhibition was relying on the dose of piperine. They showed that aflatoxin reduction was correlated to the down regulation of genes belonging to the aflatoxin's biosynthetic pathway and of gene encoding veA, a global regulator. Piperine also causes an over-expression of genes coding for several basic leucine zipper (bZIP) transcription factors such as *atfA, atfB* and *ap-1* and genes encoding enzymes belonging to superoxide dismutase and catalase's families. Hu et al. (2017) suggested that the down-regulation of the transcription level of aflatoxin biosynthetic genes, i.e. *aflM, aflO, aflP*, and *aflQ* may be responsible of the reduction in aflatoxin B1 production by *Aspergillus flavus* exposed to turmeric essential oil. El Khoury et al. (2016) showed that essential oils (fennel, cardamom, anise, chamomile, celery, cinnamon, thyme, taramira, oregano and rosemary) used principally to inhibit the production of ochratoxin A have an effect on the expression levels of the genes responsible for the ochratoxin biosynthesis in *Aspergillus carbonarius* (*acOTApks* and *acOTAnrps* along with the *acpks* gene) and the two regulatory genes *laeA* and *veA*. According to the results obtained by El Khoury et al. (2017b), the use of aqueous extract of *Micromeria graeca* (Hyssop) inhibited aflatoxin B1 synthesis. This inhibition was synchronized with 3-fold down-regulation of *aflR* and *aflS* genes, the two internal cluster co-activators, causing a radical repression of all aflatoxin biosynthesis genes. In addition, fifteen regulating genes including *veA* and *mtfA* and two major global-regulating transcription factors was impacted by the extract. The effect of Hyssop was also related to an alteration of the expression of a number of oxidative stress-defense genes such as *msnA, srrA, catA, cat2, sod1, mnsod* and *stuA*.

4.3 Mycotoxin Detoxifying Using Plants Extracts

Mycotoxins are resistant to food processing and the preservation processes because they are chemically stable and heat resistant. Various physical, chemical and biological methods have been described for mycotoxin detoxification in food (Vijayanandraj et al. 2014). In any case, each treatment suffers from some limitations. Physical methods like pressure-cooking cause the destruction of certain nutrients. Chemical methods can be a risk for the food safety due to chemical residues. The use of microorganisms, such as a biological detoxification method, is frequently used, but microorganisms use the food nutrients for their own growth and thus produce several undesirable compounds (Iram et al. 2015). Natural plants extracts can be an alternative to synthetic chemicals for detoxification of mycotoxins. Velazhahan et al. (2010) demonstrated that the use of the seed extract of Ajowan (*Trachyspermum ammi* (L.) Sprague ex Turrill) provoked the degradation of aflatoxin G1. The dialyzed *T. ammi* extract was found more effective than the crude extract, it was capable of degrading >90% of the aflatoxin G1. Another study carried out by Vijayanandraj et al. (2014) proved the effectiveness of the leaf extract of Vasaka (*Adhatodavasica* Nees) to degrade the aflatoxin B1 (≥98%) after incubation for 24 h at 37 °C.

Corymbia citriodora aqueous extracts were also tested for their detoxification potential against aflatoxin B1 and B2 (aflatoxin B1 100 µg/L and aflatoxin B2 50 µg/L). Results indicated that *C. citriodora* leaf extract was more effective to degrade aflatoxin B1 and aflatoxin B2 (95.21% and 92.95%, respectively) than *C. citriodora* branch extract, under optimized conditions (Iram et al. 2015). Aqueous extract of seeds and leaves of *Trachyspermum ammi* were also evaluated for their ability to detoxify aflatoxin B1 and B2 (aflatoxin B1: 100 µg/L and aflatoxin B2: 50 µg/ L) by *in vitro* and *in vivo* assays. Results showed that *T. ammi* seeds extract was found to be significantly effective in degrading aflatoxin B1 and aflatoxin B2 (92.8 and 91.9% respectively) (Iram et al. 2016). The treatment with *Ocimumtenui florum* extract at 85°C for 4 h led to 74.7% degradation of aflatoxin B1 (Panda and Mehta 2013). Perczak et al. (2016) studied the ability of essential oils to reduce zearalenone toxin level. They showed that the type of essential oil influenced the effectiveness of zearalenon toxin level reduction. Indeed, lemon peel *(Citrus limonum,* Italy*),* grapefruit peel (*Citrus paradisi,* Argentina*),* eucalyptus leaf oil *(Eucalyptus radiata,* China), and palmarosa leaf oil *(Cymbopogon martinii)* were the most effective, while lavender, thyme flower and leaf (*Thymus vulgaris*, Spain), and rosemary flower and leaf *(Rosmarinus officinalis,* Spain*)* did not degrade zearalenone.

5. Essential Oils Safety

Essential oils are complex mixtures of many chemical compounds among which terpenes and their oxygenated compounds are the principal components. They are usually used as aroma additives in food, pharmaceuticals and cosmetics (Von Fraunhofer and Joshi 2019). Since they are molecules of natural origin, biodegradable, they are therefore considered as a possible alternative to fungicide (El Ouadi et al. 2017). In addition to these characteristics, essential oils have proven to be effective in the treatment of many incurable diseases in humans like cancer (Hanif et al. 2019). Most essential oils and their main compounds are considered non-mutagenic or genotoxic. The negative effects generally occur at high doses and most essential oils have low acute toxicity, more than 2 g/kg for both oral and dermal application (Pavela and Benelli 2016). However, that does not mean that the use of essential oils is completely safe. Indeed, numerous studies have shown that the use of essential oils could have harmful effects on human health, such as asthma exacerbation and the decrease of pulmonary function (Levy et al. 2019). Moreover, some volatile compounds of essential oils can be classified as potentially hazardous (Nematollahi et al. 2018). That does not mean that essential oils have to be avoided but they must be used with caution and according to regulations. Essential oil safety and its regulation is of world concern (Es et al. 2017). World Health Organisation (WHO), Food and Agriculture Organisation of the United Nations (FAO), International Organisation for Standardisation (ISO), Food and Drug Administration (FDA) as well as EU Commission or American Essential Oil Trade Association (AEOTA) contribute to ensure the

control of safe and legal use of essential oils. Their use as food additives or flavourings has to be authorized by food safety authorities. For this purpose, many studies are carried out on essential oils in order to set their safety limits through evaluation of DL50 on mice or rat (Prakash et al. 2015). European Food Safety Authority (EFSA) assesses risks before allowing their use in food (Barbieri and Borsotto 2018). EU legislation regulates essential oils through the general regulations REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging of substances and mixtures). In USA, essential oils have to be recognized as GRAS (Generally Recognized As Safe) and approved by Food and Drug Administration (FDA) to be used as food additives. Although they showed low toxicity on non-target vertebrates, their use in crops as biopesticides remains low and includes regulatory requirements which can vary considerably from one country to another (Prakash et al., 2015, Isman 2016, Pavela and Benelli 2016, Dosoky and Setzer 2018, Isman 2020).

6. Conclusion

The use of plant extracts as antifungal and anti-mycotoxin agents seems to be promising and safe for human and environmental health compared to chemicals, as long as the regulatory doses established by the health authorities are respected. Many studies have shown that plant extracts inhibited mycotoxinogenesis at lower concentrations than those reducing fungal growth. Thus, their use as food bio-preservation agents for mycotoxin control in the agri-food chain is attractive because at these low concentrations, they have less impact on the organoleptic quality of foodstuffs. However, studies need to be conducted to assess consumer acceptability. Plant extracts are complex and contain many different compounds. So, data on their composition and their active molecules have still to be collected. Their absence of toxicity must be checked as well as the possible synergistic effects between the plant extracts and/or their constituents. Concerning the degradation of mycotoxins by plant extracts, chemical reactions are poorly known and studies to decipher them must continue as well as the *in vivo* evaluation of toxicity of the molecules resulting from mycotoxin degradation. The assessment of the impact of extracts on the organoleptic, technological and nutritional quality of foodstuffs must not be omitted.

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1.2 Antoferine®

1.2.1 Généralités sur le biocontrôle et l'Antoferine®

Les produits de biocontrôle sont des alternatives aux pesticides chimiques dont l'usage est de plus en plus controversé en raison de leur impact sur la santé humaine et animale ainsi que l'environnement (Rani et al., 2021). Les attentes des consommateurs en matière de santé et de respect de l'environnement sont accompagnées d'une suivies par une évolution de la réglementation en Europe et dans le reste du monde afin de réduire l'utilisation des intrants chimiques. Ce mouvement est illustré par la Directive Européenne 2009/128/EC ou encore le Plan Ecophyto 2025 en France qui vise à réduire l'usage de pesticides chimiques de 50% d'ici 2025. Il existe donc bien un besoin pour des nouvelles solutions naturelles pour la protection des végétaux.

Les produits de biocontrôle sont définis à l'article L. 253-6 du code rural et de la pêche maritime. Ce sont des agents et des produits utilisant des mécanismes naturels dans le cadre de la lutte intégrée contre les ennemis des cultures. Les substances ou produits de biocontrôle peuvent être constitués de macro-organismes, de micro-organismes (Farbo et al., 2018; Ianiri et al., 2017; Pellan et al., 2020), de médiateurs chimiques (X. Wang et al., 2019) ou de substances naturelles d'origine végétale, animale ou minérale (Enyiukwu et al., 2014)**.**

En France, la quasi-totalité des entreprises fabriquant des produits de biocontrôle appartiennent à l'Association française des fabricants de produits de biocontrôle (IBMA France). L'un des objectifs du groupe est de tripler la part de marché des produits de biocontrôle en France d'ici 2025 (Villemaine et al., 2021).

La société Antofénol (Plestan, France) développe une solution antifongique innovante d'origine naturelle pour protéger les fruits et légumes contre la contamination fongique, l'Antoferine®. A terme, elle souhaite produire et commercialiser à échelle industrielle ce produit de biocontrôle ayant des propriétés antifongiques à appliquer en pré-récolte et post-récolte pour lutter contre les maladies de conservation en alternative à l'imazalil (retrait prévu en 2024). Cet extrait est en cours d'homologation et sera commercialisé sur le marché américain en 2022 et sur le marché européen en 2024. L'efficacité du produit repose sur sa composition unique d'extraits de polyphénols, obtenus par un processus d'éco-extraction sans solvant de synthèse qui les isole à partir de co-produits de la vigne (sarments).

Avec ce projet, l'entreprise Antofénol veut contribuer à réduire les pertes post-récolte qui affectent le secteur alimentaire et tout en respectant l'économie circulaire européenne en favorisant la valorisation d'un déchet agricole abondant. Si les cultures ciblées dans un premier temps sont les bananes, agrumes, pommes, fraises, pêches, cerises, le spectre d'activité de l'Antoferine® pourrait être élargi à d'autres productions végétales, notamment celles qui sont affectées par les moisissures mycotoxinogènes.

1.2.2 *Vitis vinifera*

L'origine de la vigne se confond avec l'histoire des végétaux. Sa culture a débuté il y a environ 4000 ans à partir des espèces sauvages du proche orient (nord-est de l'Afghanistan jusqu'aux frontières méridionales de la mer Noire et de la mer Caspienne) (Aradhya et al., 2003).
La vigne appartient à la famille des *Vitaceae*, qui est divisée en différents genres (Figure 1). A l'heure actuelle, 19 genres reconnus composent cette famille, dont le genre *Vitis* (De La Fuente Lloreda, 2018). Ce genre est à son tour divisé en deux sous-genres : *Euvitis* et *Muscadinia*. Le sous-genre *Muscadinia* diffère par des caractères morphologiques, anatomiques et cytologiques de base (Galet, 1979), et comprend trois espèces. La plus connue, *V. rotundifolia,* est originaire du sud-est des États-Unis et a été domestiquée par les colons européens. Elle sert de porte-greffe pour faire face à la grande sensibilité des vignes européennes à la maladie du phylloxéra. Cette section comprend également *V. munsoniana* J.H.Simpson ex Planch. (Originaire de Floride et des Bahamas) et *V. popenoei* J.L.Fennell (du Mexique, du Belize et du Guatemala) (Fortes & Pais, 2015).
Le sous-genre *Euvitis* comprend la plupart des espèces viticoles. *Vitis vinifera*, est la principale espèce de vigne cultivée en Europe et dans le monde (Tkacz et al., 2019), avec une production mondiale de 79 millions tonnes de raisin en 2018 (https://www.oiv.int/). Elle est originaire d'Asie, près de la mer Caspienne, puis a été importée en Europe (Fortes & Pais, 2015). *Vitis vinifera* représente plus de 90% des baies de raisin sur le marché. Les baies sont consommées sous forme de fruits frais ou d'autres produits transformés, notamment le jus de raisin, la confiture, le vin, les raisins secs, le vinaigre et l'huile de pépins de raisin (Venkitasamy et al., 2019). Le *V. vinifera* doit être greffé sur le *V. rotundifolia* ou sur d'autres porte-greffes résistants pour survivre dans les régions où sévit le phylloxéra (Fortes & Pais, 2015).

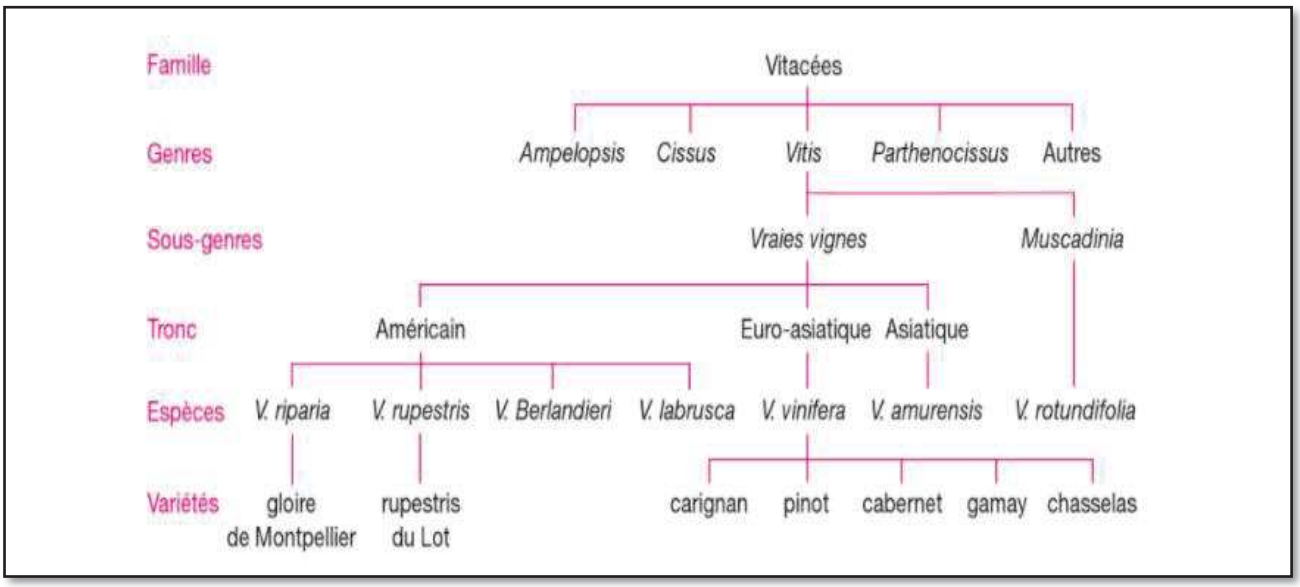

Figure 1: Schéma réduit de la famille des Vitacées (Reynier, 2011).

Morphologiquement, la vigne, comme toute plante, développe un système racinaire qui colonise le sol et le sous-sol tout au long de sa vie et un système aérien (Figure 2), formé d'un tronc qui se divise en bras portant des bois de taille qui peuvent être longs ou courts. Ces bois appelés sarments portent des yeux, ou ensembles de bourgeons qui donneront naissance à des rameaux feuillés, fructifères ou non (Reynier, 2011).

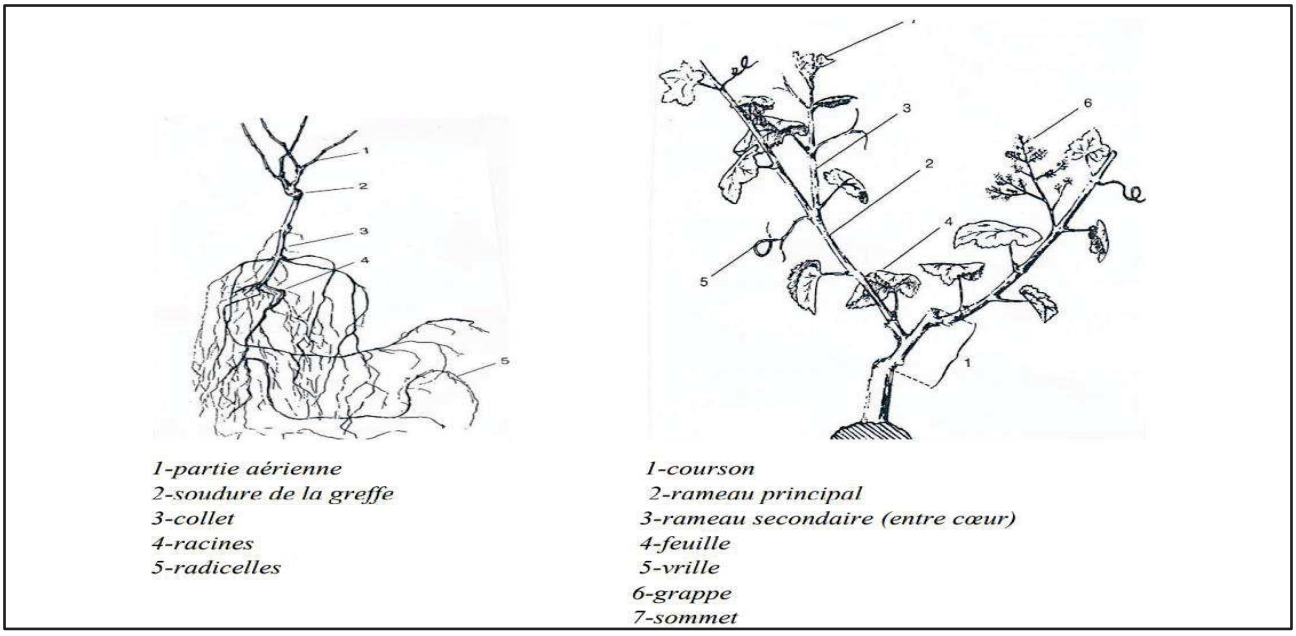

Figure 2 : Morphologie de la vigne (Hidalgo, 2008).

Le cycle annuel de développement de la vigne comprend un certain nombre de phases (Figure 3), en étroite relation avec les conditions climatiques. La période de repos végétatif de la vigne débute au mois de novembre avec la chute des feuilles et s'achève à la fin du mois de février. L'entrée en végétation se produit entre la première et la dernière semaine du mois de mars. Le nombre d'inflorescences qui apparaît après le débourrement constitue la sortie des premières feuilles. La pleine floraison a lieu généralement 2 mois après le débourrement et l'aoûtement, comme son nom l'indique, débute au mois d'août et se poursuit jusqu'en novembre. Il s'accompagne d'une accumulation de réserves en amidon et en lignine dans les sarments, ce qui favorise la résistance des tissus au froid et permettra le début d'un nouveau cycle de développement au printemps suivant (Galet, 2000 ; Carbonneau et al., 2020).

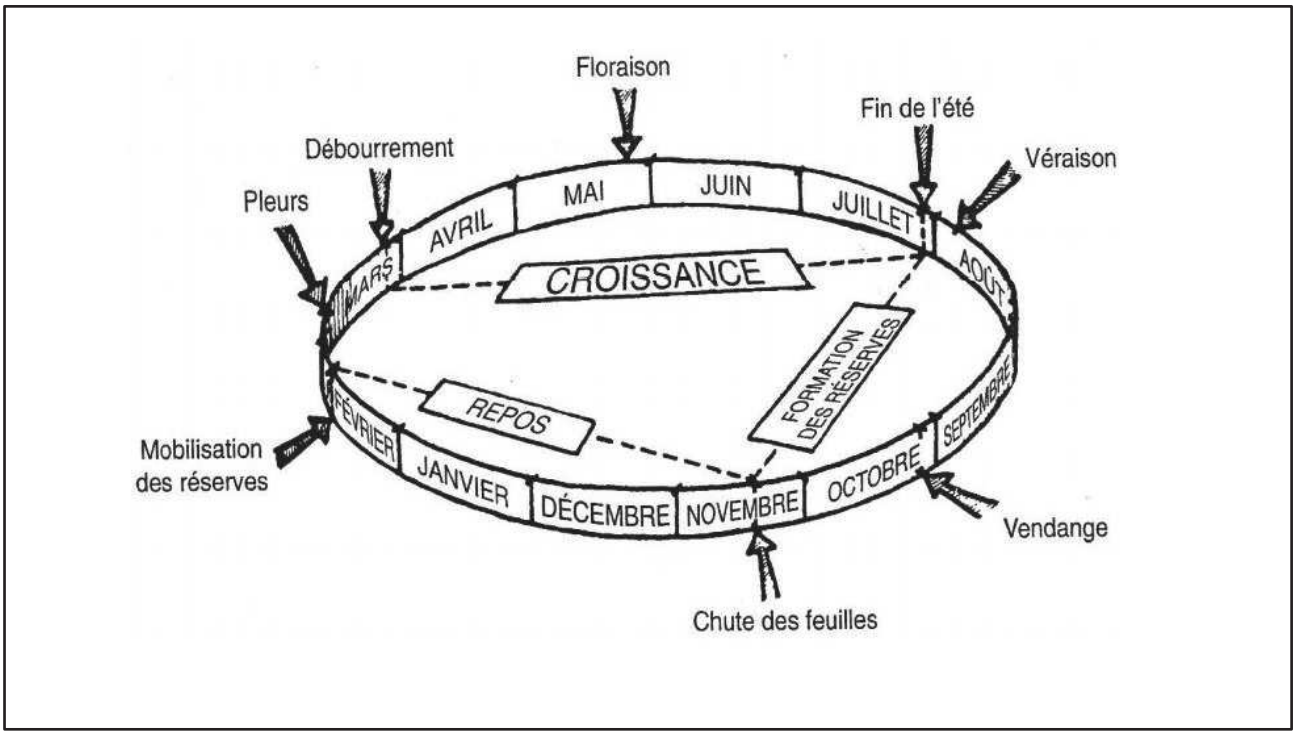

Figure 3: Cycle végétatif de la vigne applicable aux régions de l'hémisphère Nord (Hidalgo, 2008).

1.2.3 Valorisation des différents organes de la vigne

La production de raisins est l'une des principales activités économiques du secteur agroalimentaire, avec plus de 79 millions de tonnes produites dans le monde en 2018 (https://www.oiv.int/). Parmi celles-ci, environ 47% sont des raisins destinés à l'industrie vinicole ("The International Organisation of Vine and Wine", 2017), où de grandes quantités de sous-produits sont générées en tant que déchets organiques (marc de raisin, contenant les graines, la pulpe et les peaux, les tiges et les feuilles de raisin), d'émission de gaz (CO_2, composés organiques volatils, etc.), et déchets inorganiques (terre de diatomées, bentonite et argile perlite) (Gouvinhas et al., 2019; Soceanu et al., 2021).

Généralement, après vinification, les déchets de raisin sont utilisés comme source de production de polyphénols qui sont surtout utilisés dans le domaine médical à cause de leur effet antioxydant. Parmi eux, les anthocyanes sont utilisés comme colorants naturels. En effet, ces pigments sont responsables des couleurs, comme par exemple l'orangé, le rouge, le bleu et le violet (Vatai et al., 2009).

Les tiges de raisin, qui correspondent au support des raisins, ont été principalement étudiées comme source polyphénolique ou comme source de monosaccharides pour la production de bioéthanol (Soceanu et al., 2021).

L'extrait et l'huile de pépins de raisin ont démontré de nombreuses activités dans la prévention des maladies. Certains constituants présentent des activités antioxydantes et anti-inflammatoires remarquables : les acides gras essentiels présents dans l'huile comme l'acide linoléique, la vitamine E et les phytostérols, ainsi que les phénols hydrophiles. D'autres industries non alimentaires peuvent également bénéficier de l'huile de pépins de raisin, notamment les produits pharmaceutiques et les cosmétiques (Martin et al., 2020).

Lorsque le vignoble est trop vieux ou que l'arrachage est nécessaire pour une raison quelconque, le bois et les racines de la vigne constituent également une énorme source de déchets à faible valeur ajoutée et à forte teneur en stilbènes. Une étude réalisée par Gabaston et al. (2017) montre que les déchets de la vigne tels que le bois et les racines de *Vitis vinifera* peuvent être utilisés comme sources bon marché de stilbènes bioactifs pour développer des fongicides naturels car ils contiennent quatre composés parmi les plus actifs (R-viniférine, R2-viniférine, hopeaphénol et iso-hopeaphenol) à des niveaux élevés.

Les déchets comme les feuilles de vigne, les tiges, les pépins, les marcs, les lies de vin, les vinasses, sont aussi utilisés pour la biosynthèse de divers bioproduits à haute valeur ajoutée, à

savoir les acides hydroxybenzoïques, les acides hydroxycinnamiques, les flavonoïdes, les acides tartriques ou les substrats lignocellulosiques (Ahmad et al., 2020).

1.2.4 Méthodes d'extraction de l'Antoferine® à partir des co-produits de *Vitis vinifera*

L'utilisation des composés bioactifs dans différents secteurs commerciaux tels que les industries pharmaceutiques, alimentaires et chimiques nécessite l'obtention d'extraits de qualité maîtrisée. Ainsi, il est nécessaire de disposer de la méthode la plus appropriée et la plus standardisée possible permettant d'extraire ces composés actifs des matériaux végétaux (Azmir et al., 2013). Les molécules d'intérêt peuvent être récupérées à partir de la source végétale en ayant recours à l'extraction solide-liquide. Des procédés classiques sont couramment utilisés pour l'extraction des composés mais ces opérations requièrent généralement l'utilisation de solvants toxiques, des températures élevées, pendant des temps de traitement longs, entraînant ainsi des consommations d'énergie importantes (Ameer et al., 2017).

Dans un contexte de développement durable, ces opérations doivent être améliorées afin de proposer un produit sûr, issu de technologies permettant la diminution des impacts environnementaux. C'est dans ce cadre que l'entreprise Antofénol a développé une méthode d'éco-extraction pour récupérer des polyphénols de qualité à partir des différentes parties des plantes du genre *Vitis (Vitis labrusca, Vitis riparia, Vitis rupestris, Vitis berlandieri, Vitis amurensis, Vitis coignetiae, Vitis vulpina, Vitis acerifolia, Vitis aestivalis, Vitis rotundifolia)* et plus particulièrement de *Vitis vinifera.* Cette méthode d'extraction a été mise au point par Fanny Rolet et Ludovic Fabre de la société Antofénol et est décrite dans le brevet afférent déposé auprès de l'INPI (FR3061176A1, 2016).

Pour la biomasse, l'entreprise utilise des plantes entières ou des parties de plantes de vigne, plus particulièrement des parties aériennes des plantes comme les fruits, les fleurs, les feuilles, les tiges, les branches ou des fragments de troncs (ceps) et leurs mélanges. Le cas échéant, la biomasse peut aussi comprendre ou être constituée de racines de plantes. La biomasse utilisée pour l'extraction est sèche. Elle est obtenue par séchage des plantes ou des parties de plantes précitées. Le séchage est réalisé en laissant les plantes ou leurs parties sécher à l'air libre. Le séchage va d'un mois à quatre ans, de préférence d'un à deux ans.

Une fois sèches, les plantes et leurs parties sont d'abord déchiquetées en fragments de 1 à 20 cm puis broyées finement à une granulométrie inférieure ou égale à cinq mm. La poudre

obtenue est mise en contact avec un solvant qui est un mélange d'alcool et d'eau, en particulier éthanol/eau. Avantageusement, le rapport en masse biomasse végétale sèche/solvant aqueux va de 1/5 à 1/30, préférentiellement de 1/10 à 1/20.

Le procédé de préparation d'un extrait de biomasse végétale sèche (Antoferine®), en particulier un extrait de *Vitis* sp. riche en polyphénols, comprend donc :

(a) une étape d'extraction de la biomasse sèche par sa mise en contact avec un solvant aqueux

(b) une étape de récupération de la phase aqueuse enrichie en polyphénols : le mélange biomasse végétale / solvant aqueux est traité de manière simultanée ou séquentielle par la combinaison de (i) des ondes électromagnétiques de fréquence allant de 915 MHz à 28 GHz et (ii) un brassage du mélange et/ou (iii) une pression de 50 à 950 mbars (5 000 à 95 000 Pa).

A la fin de l'extraction, il y a une étape additionnelle qui est la concentration des polyphénols obtenue par évaporation partielle ou totale du solvant aqueux. Cette concentration se fait par lyophilisation de la phase aqueuse récupérée.

L'élimination des sucres, selon la biomasse traitée, est importante pour l'obtention d'un extrait riche en polyphénols aux propriétés antifongiques. En effet, la présence de sucres pourrait avoir un effet antagoniste à l'activité antifongique des polyphénols en favorisant la croissance des champignons. Pour cela, avant l'étape d'extraction, la biomasse séchée est soumise préalablement à une lixiviation par un solvant approprié pour éliminer les sucres.

L'extrait sec ainsi obtenu est ensuite stocké à température ambiante à l'abri de la lumière.

1.2.5 Les composés majeurs de l'Antoferine®

Les polyphénols sont les métabolites secondaires les plus abondants du règne végétal (Ghani, 2020 ; Kundu et al., 2019). Il existe environ 8000 composés (Chandrasekara & Shahidi, 2018) et ils arrivent en troisième position dans la composition des raisins, après les glucides et les acides (Nawaz et al., 2006). Ils sont considérés comme des phytoalexines de structure chimique C6-C2-C6 (Kiselev et al., 2019). Les polyphénols sont synthétisés généralement par les plantes en tant que composés de défense contre les stress biotiques et abiotiques (Cacho et al., 2013). Ils sont associés à la résistance des plantes aux maladies et leur synthèse est souvent due à une réponse aux attaques par des agents phytopathogènes et d'autres facteurs de stress comme l'irradiation UV (Błaszczyk et al., 2019) . Parmi les polyphénols du raisin, les stilbènes peuvent

être sous forme de monomères, dimères ou oligomères. Ils peuvent subir plusieurs modifications (glycosylation, méthoxylation, oligomérisation et isoprénylation) pour donner des composés plus complexes (Chong et al., 2009). Les stilbènes monomères les plus abondants dans les baies sont le trans-resvératrol et le trans-picéide (Błaszczyk et al., 2019). L'Antoferine® est un produit riche en polyphénols dont les principaux sont les trans-picéatannol, trans-resvératrol, trans-Ɛ-viniférine et trans-vitisine. De manière générale, l'extrait contient au moins deux fois plus de trans-Ɛ-viniférine (voire 2,5 fois plus) et au moins trois fois plus de trans-vitisine (voire six fois plus) qu'un extrait éthanolique conventionnel.

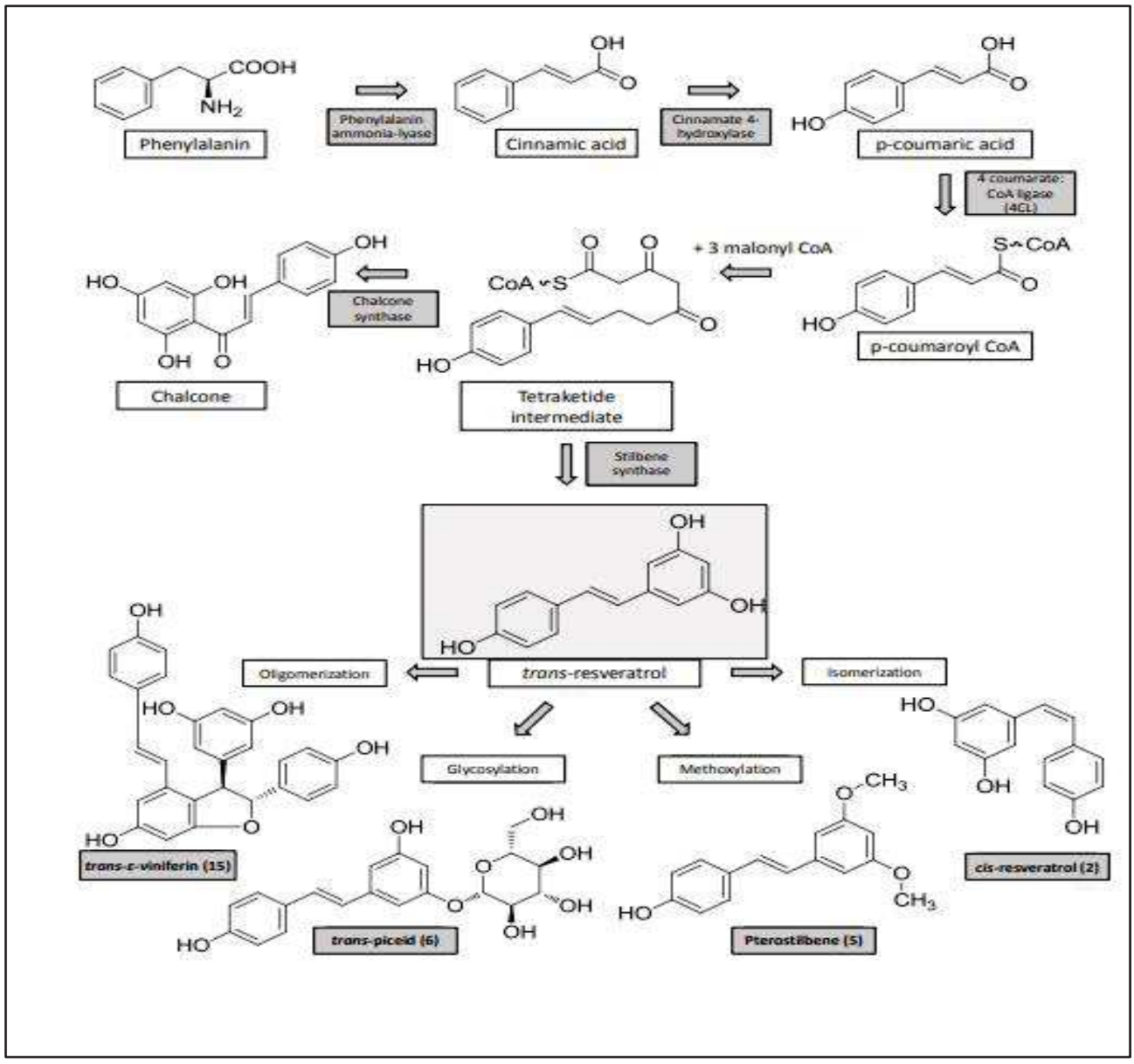

Figure 4: Biosynthèse du trans-resvératrol et ses voies de diversification chimique (Benbouguerra et al., 2021).

1.2.6 Activités biologiques et antifongiques des extraits de la vigne et de l'Antoferine®

L'Antoferine® a montré des propriétés antioxydantes pouvant être utilisées pour la préservation de compositions cosmétiques ou pour la prévention et le traitement du stress oxydatif chez l'homme et l'animal. Une activité antibactérienne, en particulier sur les bactéries gram+ pathogènes, tout en préservant les bactéries commensales de l'homme, a également été mise en évidence. L'Antoferine® peut également être utilisé pour contrôler le développement de champignons nuisibles dans l'alimentation et en agriculture, comme les contaminants de fruits en conserve (*Byssochlamys nivea*), ou des maladies des arbres fruitiers, des légumes et des plantes, comme le chancre du pommier (*Nectria galligena*), la moniliose des arbres fruitiers (*Monilinia fructicola, Monilinia fructigena et Monilinia laxa*), l'alternariose de la carotte (*Alternaria dauci*), labnbv galle argentée de la pomme de terre (*Helminthosporium solani*), la fusariose du maïs (*Gibberella zeae*), la fusariose du blé (*Fusarium culmorum*), la septoriose du blé (*Mycosphaerella graminicola*) ou différentes pourritures (notamment *Penicillium digitatum, Penicillium italicum, Phytophthora syringae* ou *Rhizopus stolonifer).*

L'application de l'Antoferine® sur les plantes se fait soit à titre préventif, lorsque les conditions climatiques sont favorables au développement des champignons, soit à titre curatif après apparition des maladies. L'application se fait généralement selon des méthodes usuelles de pulvérisation et le produit peut être employé seul ou en mélange avec d'autres produits connus pour leurs propriétés fongicides, comme le sulfate de cuivre, la bouillie bordelaise ou d'autres produits phytosanitaires issus de l'industrie agrochimique. De manière avantageuse, le traitement est fait sans employer de produits phytosanitaires de l'industrie agrochimique, pour une agriculture biologique ou biodynamique. L'extrait peut être ainsi utilisé pour prévenir et/ou traiter la dégradation des fruits et des légumes après récolte, en particulier pour les fruits sensibles à l'oxydation comme la banane ou les fruits exotiques ou tropicaux comme la mangue ou les fruits de la passion qui sont sensibles au stress oxydatif lorsqu'ils sont conservés à des températures basses, plus basses que celles rencontrées dans leur environnement naturel.

1.3 Huiles essentielles

1.3.1 Généralités

Contrairement à ce que le terme pourrait laisser penser, les huiles essentielles ne contiennent pas de corps gras comme les huiles végétales obtenues avec des pressoirs (huile de tournesol, de maïs, d'amande douce, etc.). Ce sont des fractions liquides concentrées, liposolubles et hydrophobes obtenues par extraction de matériel végétal. Les huiles essentielles sont des extraits composés de molécules aromatiques provenant des plantes aromatiques ou de leurs organes (Baudoux, 2017). Elles n'existent quasiment que chez les végétaux supérieurs et il y aurait environ 17500 espèces aromatiques. Les genres capables d'élaborer les constituants qui composent les huiles essentielles sont répartis dans certaines familles telles que les *Apiaceae, Asteraceae, Lamiaceae, Lauraceae, Myrtaceae, Rutaceae, Poaceae* (Baudoux, 2017; Bruneton, 2009)**.**

Les huiles essentielles sont produites par des cellules végétales spécialisées et peuvent être stockées dans tous les organes végétaux comme les feuilles (eucalyptus, citronnelle, laurier noble), les fleurs (camomille, lavande), les zestes (citron, orange, bergamote), le bois (bois de rose, santal), la racine (vétiver), l'écorce (cannelle), les fruits (anis, badiane), les rhizomes (curcuma, gingembre) ou encore les graines (muscade) (Baudoux, 2017). La synthèse et l'accumulation des huiles essentielles sont généralement associées à la présence de structures histologiques spécialisées souvent localisées sur ou à proximité de la surface de la plante. Ce sont les cellules à huiles essentielles chez les Lauracées et les Zingiberacées, les poils sécréteurs chez les Lamiacées, les poches sécrétrices chez les Myrtacées et les Rutacées, les canaux sécréteurs chez les Apiacées et les Astéracées (Aziz et al., 2018; Bruneton, 2009)

Elles possèdent une valeur olfactive et gustative fortement aromatique. Elles sont obtenues par distillation ou par extraction chimique par des solvants (eau, alcool ou autre). Pour la Pharmacopée européenne (6e édition) (Tomi et al., 2008), l'huile essentielle est un produit odorant, obtenu à partir d'une matière première végétale botaniquement définie. Le plus souvent, elle est séparée de la phase aqueuse par un procédé physique n'entraînant pas de changement significatif dans sa composition.

La quantité d'huile essentielle contenue dans les plantes est souvent faible, parfois très faible, voire infime, ce qui explique leur coût élevé. Il faut parfois plusieurs tonnes de plantes pour obtenir un litre d'huile essentielle. D'une manière générale, les huiles essentielles sont des

mélanges complexes de constituants appartenant à différentes familles chimiques. Les plus couramment représentées sont les terpénoides et les composés aromatiques, mais on y trouve également des constituants aliphatiques issus de la synthèse des acides gras et plus rarement d'autres composants d'origines diverses (dérivés soufrés, nitriles, thiocyanates…) (Baudoux, 2017; Bhardwaj et al., 2020). Les constituants principaux des huiles essentielles sont les monoterpènes et les sesquiterpènes, les glucides, les phénols, les alcools, les éthers, les aldéhydes et les cétones, responsables de l'activité biologique ainsi que de leur parfum. Les composés phénoliques présents dans les huiles essentielles ont également été reconnus comme composants bioactifs antimicrobiens (Baudoux, 2017)**.**

Les huiles essentielles se différencient des huiles grasses, par leurs propriétés physiques et leur composition (Baudoux, 2017; Bruneton, 2009) du fait qu'elles sont liquides à température ambiante, n'ont pas le toucher gras et onctueux des huiles végétales, volatiles et très rarement colorées, de densité faible pour les huiles essentielles à forte teneur en monoterpènes. Leur indice de réfraction varie essentiellement avec la teneur en monoterpènes et en dérivés oxygénés. Une forte teneur en monoterpènes donnera un indice élevé, alors qu'une teneur élevée en dérivés oxygénés produira l'effet inverse. Elles sont solubles dans les alcools à titre alcoométrique élevé et dans la plupart des solvants organiques mais peu solubles dans l'eau, dotées d'un pouvoir rotatoire car elles sont formées principalement de composés asymétriques et également très altérables, sensibles à l'oxydation et ont tendance à se polymériser donnant lieu à la formation de produits résineux. C'est pour cela qu'il convient de les conserver à l'abri de la lumière et de l'air.

En fait, les essences produites par différentes espèces de plantes varient dans leurs caractéristiques physico-chimiques selon plusieurs facteurs. L'espèce botanique (Sangwan et al., 2001), le chémotype (Deschepper, 2017), l'âge de la plante, le cycle végétatif, la période de récolte, l'organe végétal, les facteurs extrinsèques (Barra, 2009; Jugreet et al., 2020; Tanoh et al., 2020; Walia et al., 2020) et les procédés d'obtention sont les principaux facteurs qui peuvent influencer à la fois la composition chimique proprement dite et le rendement de leur extraction (Miljanović et al., 2020).

Les molécules constituant les huiles essentielles étant souvent instables, leur conservation est difficile et les possibilités de dégradation sont nombreuses. Ainsi, les propriétés du produit sont aisément modifiées. Pour éviter l'altération, sont utilisés des flacons propres et secs, en aluminium, en acier inoxydable ou en verre teinté antiactinique et pour éviter l'évaporation, ceux-ci sont presque entièrement remplis et scellés. La conservation des flacons se fait à une

température comprise entre 5°C à 35°C. Les huiles essentielles pures et naturelles se conserveront ainsi pendant plusieurs années. Il est à noter que certaines huiles, telles que les essences de citrus, se conservent moins longtemps (Baudoux, 2017).

1.3.2 Procédés d'extraction des huiles essentielles

Il existe plusieurs méthodes d'extraction des huiles essentielles. Ces dernières ont un impact sur leurs qualités et leurs applications. En effet, la localisation histologique des composés aromatiques dans le végétal, ainsi que la destination finale du produit extrait peuvent orienter le choix technologique. Les méthodes d'extraction sont adaptées aux propriétés les plus importantes des huiles essentielles (leur volatilité dans l'air, dans la vapeur d'eau ainsi que leur solubilité dans les solvants organiques). La composition des huiles essentielles peut varier selon le mode d'extraction utilisé (distillation, hydrodistillation ou expression). La fragilité des constituants des huiles essentielles explique la différence entre la composition du produit obtenu, par hydrodistillation, et celle du mélange initialement présent dans les organes sécréteurs du végétal.

1.3.2.1 L'extraction à froid ou expression mécanique à froid

L'avantage de la méthode de pression à froid est qu'elle est simple, naturelle, sûre, écologique, traditionnelle, moins coûteuse et n'implique aucune chaleur (Bhardwaj et al., 2020). La plupart des huiles essentielles d'agrumes sont extraites par ce procédé qui consiste à soumettre la matière à une grande pression mécanique (écorces ou zestes, de bergamote, citron, lime, mandarine, orange) car l'huile essentielle d'agrumes est présente dans des sacs d'huile ou des glandes huileuses situés à différentes profondeurs dans la peau et les cuticules du fruit (Russo et al., 2020; Teigiserova et al., 2021)

La qualité des huiles essentielles extraites à froid est élevée car elle n'a pas besoin d'être raffinée. D'autres avantages de cette technique sont que divers composés phénoliques sensibles à la température, des composés phytochimiques lipophiles tels que les antioxydants, ne sont pas perdus, ce qui améliore la durée de conservation de l'huile (Ramadan, 2020). Cependant, le processus de purification comprend le lavage à l'eau, la filtration et la centrifugation. Le principal inconvénient est le faible rendement et la qualité du produit qui peut varier. En outre, il est parfois nécessaire de procéder à un prétraitement, comme le séchage et l'épluchage. Une

autre difficulté constatée est qu'il est difficile de conserver les produits pendant une longue période. La durée de vie est courte, inférieure à six mois (Bhardwaj et al., 2020).

1.3.2.2 La distillation

Ce procédé utilise la nature volatile des composants aromatiques pour les séparer du reste de la plante. Il existe deux formes de distillation :

L'hydrodistillation ou distillation simple

C'est la technique la plus simple et la plus répandue (Figure 5). Elle consiste à immerger la matière première végétale directement dans l'eau, puis l'ensemble est porté à ébullition. L'opération est généralement conduite à pression atmosphérique. Les vapeurs formées sont condensées par un système de réfrigération par courant d'eau puis récupérées en fin de parcours dans un vase à décanter. Cette méthode est le moyen le plus facile d'extraire les huiles essentielles car elle prend moins de quatre heures. Par contre, la mise en contact de l'eau et du végétal pendant la chauffe favorise l'altération des composés aromatiques, particulièrement des esters (Bhardwaj et al., 2020; Bruneton, 2009).

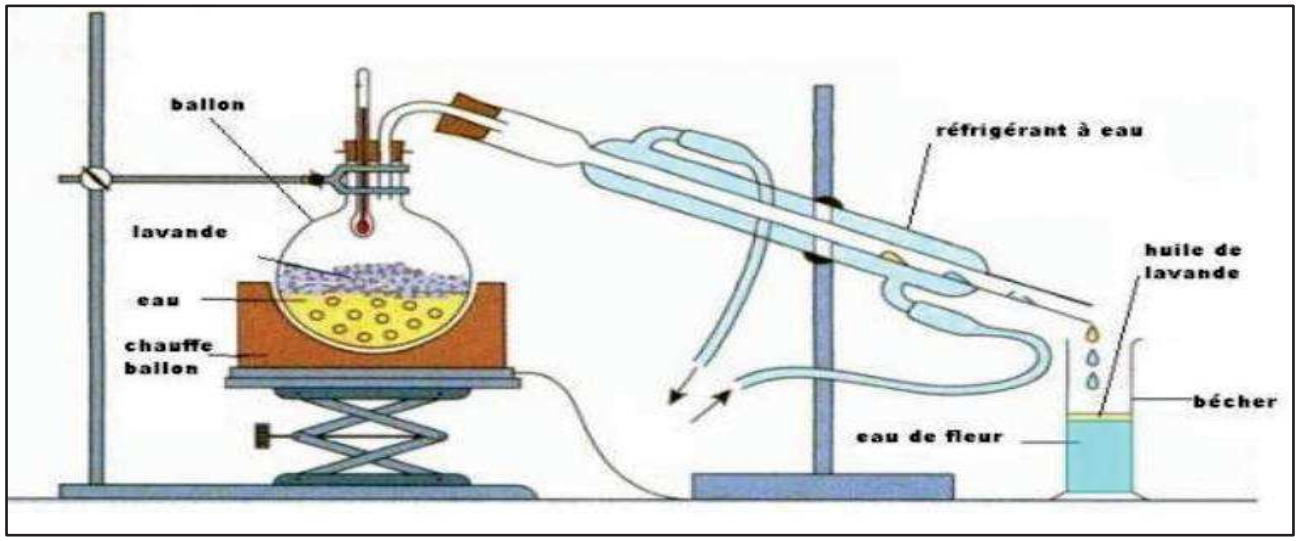

Figure 5: Principe de la distillation simple (https://www.physicsforums.com/threads/hydrodistillation-and-original-uses).

L'entrainement à la vapeur d'eau

La vapeur d'eau est pulsée de bas en haut à travers la masse végétale (Figue 6), disposée sur des plaques perforées. Les cellules se relâchent et les particules d'huile se libèrent. Le mélange vaporeux ainsi formé va être condensé à l'aide d'un réfrigérant à eau.

Les produits de la distillation sont l'huile essentielle et l'hydrolat aromatique. Ces deux produits sont séparés par simple décantation, l'huile essentielle étant en règle générale plus légère que l'eau. L'appellation d'huile essentielle (plutôt qu'essence) se justifie par les transformations que subit l'essence primitive au cours du processus de distillation : oxydation, hydrolyse…(Baudoux, 2017 ; Bhardwaj et al., 2020)

L'eau résiduelle est parfois commercialisée sous l'appellation « eau florale », « hydrosol » ou « hydrolat ».

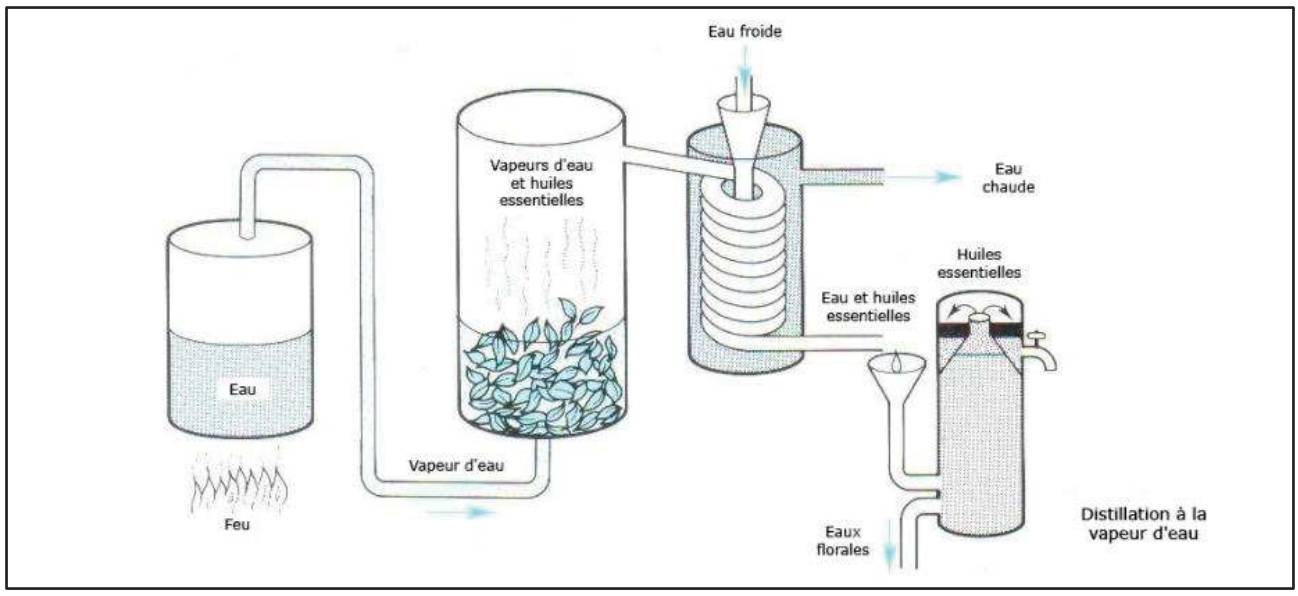

Figure 6 : Principe de la distillation par l'entraînement de la vapeur (http://www.rosemarycreek.com/img/cms/Distillation_francais.jpg).

1.3.2.3 L'hydrodiffusion

À l'image de la distillation par entraînement à la vapeur d'eau, ce procédé utilise la vapeur d'eau pour entraîner les composés d'intérêt mais, à l'inverse de la distillation, la vapeur est ici injectée de haut en bas, à faible pression (entre 0,02-0,15 Bar) à travers la masse végétale. Les huiles essentielles, obtenues par distillation à la vapeur, laissent de la matière végétale résiduelle qui

peut être valorisée. Ces plantes, en plus des huiles essentielles, contiennent également des flavonoïdes, qui pourraient être extraits comme démontrés pour d'autres sous-produits de l'industrie des huiles essentielles (Arias et al., 2020 ; Bruneton, 2009). L'hydrodiffusion permet une extraction plus rapide et moins coûteuse en énergie, mais au détriment de la qualité du produit final.

1.3.2.4 L'extraction par solvant

Les solvants tels que l'éther de pétrole, le méthanol, l'éthanol ou l'hexane sont utilisés pour extraire l'huile essentielle d'éléments végétaux qui ne supportent pas la chaleur. C'est par exemple le cas de la jacinthe ou du jasmin. Extraite par solvant, l'huile essentielle est très concentrée, mais ce procédé n'a pas la faveur du public qui craint de retrouver des traces de solvant dans le produit final. En plus, au stade final d'extraction, il y a des pertes, telles que les constituants évaporatifs lors de l'évaporation du solvant, une faible cohérence de l'isolement (mélange de différents constituants végétaux) et possibles résidus de solvants toxiques (Baptista-Silva et al., 2020 ; Bhardwaj et al., 2020).

1.3.2.5 L'enfleurage

C'est une méthode longue et complexe qui utilise une matière grasse végétale ou animale purifiée sur laquelle est placé un lit de pétales de fleurs (Figure 7). Au bout de quelques jours de contact « corps gras/pétales de fleurs », on procède à un pressage du mélange, ce qui produit une matière huileuse très aromatique, puis à un « rinçage » de la pommade d'enfleurage (composée de gras et de parfum) avec de l'alcool. Lorsque l'alcool s'est évaporé du mélange, il ne reste que l'huile essentielle. Les huiles essentielles se dissolvent dans l'alcool sans la graisse (Eltz et al., 2007). Il existe deux types d'enfleurage : enfleurage à froid et enfleurage à chaud. Cette méthode d'extraction est à l'origine de la fabrication des grands parfums qui ont fait la renommée de la parfumerie française et ses fragrances de renommée olfactive mondiale.

Figure 7 : Technique d'enfleurage du jasmin sur une plaque enduite de graisse *(*https://www.quintessence-lab.com/wp-content/uploads/2020/05/Session-studio-075-600x600.jpg*)*.

1.3.2.6 Extraction par micro-ondes.

Les techniques d'extraction assistée par micro-ondes ont été utilisées pour extraire les principaux composants des matrices environnementales biologiques et géologiques. Au milieu des années 80, cette technique est utilisée pour isoler les composés bioactifs des plantes (Bagade & Patil, 2021).

Plus récemment, cette technique a été mise en œuvre pour extraire les huiles essentielles en un temps court et avec un rendement relativement élevé. Ce procédé, très rapide et consommant peu d'énergie et d'eau, consiste à chauffer sélectivement la plante par un rayonnement micro-ondes, dans une enceinte dont la pression est réduite, de façon séquentielle. Le produit obtenu est de qualité supérieure à celle du produit de l'hydrodistillation traditionnelle (Bhardwaj et al., 2020 ; Bruneton, 2009 ; Teigiserova et al., 2021).

1.3.2.7 L'extraction au CO_2 supercritique

Les méthodes modernes d'extraction comprennent le procédé assisté par l'extraction par fluide supercritique (Figure 8), qui peuvent présenter de grands avantages tels qu'une oxydation moindre des composés, une caractéristique particulièrement utile pour les parfums de fleurs.

L'extraction par fluide supercritique est certainement l'application la plus étudiée. Cette méthode est généralement réalisée à l'aide de dioxyde de carbone (CO_2) pour plusieurs raisons pratiques : le CO_2 a une pression critique (74 Bar) et une température critique (32 ºC) modérément basses, il est non toxique, ininflammable, disponible en grande pureté à un coût relativement faible et il est facilement éliminé de l'extrait. Les extraits végétaux supercritiques font l'objet de recherches intensives en tant que sources potentielles d'ingrédients fonctionnels naturels en raison de leurs effets favorables sur diverses maladies humaines, avec une application conséquente dans la production de nouveaux aliments fonctionnels, nutraceutiques et produits pharmaceutiques. Il est généralement admis que les extraits supercritiques se sont avérés être de qualité supérieure, c'est-à-dire avoir une meilleure activité fonctionnelle par rapport aux extraits produits par hydrodistillation ou en utilisant des solvants liquides (Baptista-Silva et al., 2020 ; Teigiserova et al., 2021).

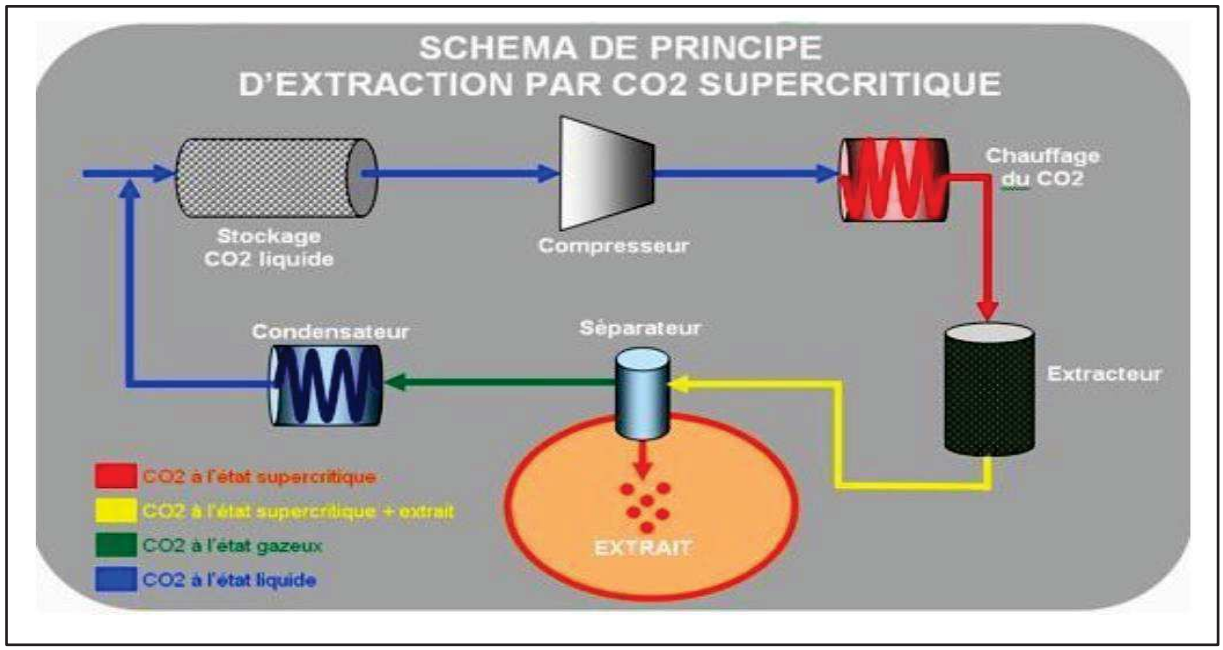

Figure 8 : Principe d'extraction par CO2 supercritique (http://www.biostimulants.fr/wp-content/uploads/2019/02/schema-1024x576.jpg).

1.3.3 *Cymbopogon schoenanthus*

1.3.3.1 Présentation de la plante et description botanique

Le genre *Cymbopogon* appartient à la famille des Poaceae et possède plus de 100 espèces dans les pays tropicaux (Likibi et al., 2019) qui se développent dans les zones tropicales et subtropicales d'Asie et d'Afrique du nord**.**

Les espèces du genre *Cymbopogon*, sont des plantes vivaces en touffes denses (Figure 8). L'épillet est sessile sans nervures intracarinales ou à deux nervures, mais sans fossette. L'inflorescence est en panicule enveloppée d'une spathe et comportant des paires de grappes pourvues d'une spathéole à la base. Les épillets géminés sont par paires et différents : un sessile et aristé avec un lemme et une fleur fertile (la fleur fertile est parfois remplacée par une neutre ou mâle dans les épillets inférieurs de la grappe) et un pédonculé et sans arête avec deux fleurs neutres ou une fleur neutre et une (supérieure) mâle (Baudoux, 2017)

Les espèces de *Cymbopogon* sont bien connues comme source de composés de valeur commerciale tels que le géraniol, l'acétate de géranyle, le citral (néral et géranial), le citronellal, la pipéritone, l'eugénol qui sont soit utilisés tels quels en parfumerie et dans les industries connexes, soit comme matières premières pour la synthèse d'autres produits couramment utilisés en parfumerie (Cos, 1980).

Cymbopogon schoenanthus (Figure 9) est une espèce endémique commune dans tout le Sahara, largement répandue dans les régions tropicales, en Afrique du Nord et en Asie. On la trouve également en Ethiopie, en Egypte, en Arabie Saoudite et dans l'Asie désertique. Elle pousse par pieds isolés dans les régions désertiques, semi-désertiques, ou semi-arides où les précipitations sont faibles (environ 100-150 mm par an). Cette plante se trouve sur les sols sablo-graveleux des lits d'oueds non salins ainsi que dans les ravins des montagnes entre 800 et 2100 m (Ozenda, 1991 ; Sahki & Sahki, 2004).

Figure 9 : Une vue complète de *Cymbopogon schoenanthus* (https://antropocene.it/2020/11/30/cymbopogon-schoenanthus/)

1.3.3.2 Huile essentielle de *Cymbopogon schoenanthus*

Composition chimique

La composition chimique de l'huile essentielle de l'espèce *Cymbopogon schoenanthus* a été largement étudiée, révélant une grande variabilité des ces composés ainsi que de leurs teneurs, généralement caractérisée par des pourcentages élevés de composés monoterpéniques, tels que la pipéritone, le 2-carène et le limonène. L'huile essentielle de l'espèce *C. schoenanthus* a été analysée par Shahi & Tava (1993)**,** sur un échantillon de feuilles récoltées en Inde. Le limonène (19,54%) est le composé majoritaire, suivi du camphène (7,98%). D'autres monoterpènes sont présents en quantités appréciables, tels les α-pinène (2,86%), myrcène (2,52%), bornéol (2,13%) et α-terpinéol (2,68%). L'élémol (4,52%), l'αcadinol (3,38%), le τ-cadinol (2,74%) et le τ-muurolol (2,20%) sont les principaux sesquiterpènes oxygénés présents en teneurs appréciables. Ces auteurs ont pu isoler et identifier également une série de cétones méthylées telles que le 2-nonanone (2,58%), le 2-undécanone (14,68%) et le 2-tridécanone (3,77%). Etonnamment, certains auteurs rapportent des compositions radicalement différentes. L'huile essentielle de *C. schoenanthus* originaire du Brésil a été analysée par Katiki et al., (2011, 2012). Le géraniol (59,42% ; 62,5%), le géranial (13,49% ; 12,5%) et le néral (8,98% ; 8,2%) sont les principaux composés, suivis du citronellol (3,18% ; 3,8%), de l'acétate de géranyle (4,80% ; 2,0%) et du E-caryophyllène (2,19% ; 3,4%). L'huile essentielle des feuilles et des inflorescences de *C. schoenanthus* récoltées au Burkina Faso est caractérisée par une forte teneur en 1,8-cinéole (50,2%), suivi du camphre (13,5%). D'autres constituants sont présents

en quantités appréciables : α-terpinéol (6,5%), β-pinène (5,7%), trans-α-bergamotene (2,8%), α-pinène (2,7%), δ-cadinène (2,5%), myrcène (2,1%), limonène (1,9%), β-caryophyllène (1,9%), sabinène (1,5%), cis-p-menth-2- en-1-ol (1,4%), terpinéol-4 (1,3%). En ce qui concerne la composition des huiles essentielles des parties aériennes de *C. schoenanthus* récoltées en Inde, les composés majoritaires identifiés sont : cis-hydrate de sabinène (30,1 et 31,6%), trans-hydrate de sabinène (17,0 et 14,5%), cis-pipéritol (9,6 et 18,5%), α-terpinolène (11,0 et 7,9%), trans-pipéritol (8,5 et 7,2%), pipéritone (2,7 et 3,7%), limonène (3,2 et 3,8%) et p-cymène (4,5 et 4,3%) (Andola & Maithani, 2018). Récemment, Aous et al., (2019) ont étudié la composition chimique de l'huile essentielle des parties aériennes de *C. schoenanthus* récoltées dans six régions du Sahara algérien (Béchar, Ghardaïa, El Menia, Ouargla, Djanet et Tamanrasset). L'analyse statistique a montré l'existence de trois groupes. Les composés majoritaires du groupe I sont : 2-carène (13,4-20,4%), limonène (8,7-13,0%), cis-p-menth-2-en-ol (12,1-15,1%), trans-p-menth-2-enol (8,4-9,2%), cis-pipéritol (3,3-6,9%), trans-pipéritol (5,4-6,6%), élémol (4,9-9,6%) et β-eudesmol (2,8-9,6%). Le groupe II (échantillon de Ouargla) contient les mêmes composés majoritaires que le groupe I à l'exception du limonène qui est totalement absent dans cet échantillon. Ce groupe diffère par sa teneur non négligeable en β-phellandrène (jusqu'à 8,0%) qui est totalement absent dans le groupe I. En revanche, les échantillons du groupe III (Djanet et Tamanrasset) sont très riches en pipéritone (55,1-63,2%), suivie de 2-carène (9,3-16,7%) et de l'élémol (4,8-9,5%).

Activités biologiques des huiles essentielles de *Cymbopogon schoenanthus*

Certaines activités biologiques ont été rapportées pour les huiles essentielles de l'espèce *Cymbopogon schoenanthus*, telles que les activités antioxydantes, antidiabétiques et atténuation de la résistance à l'insuline, activités antidiarrhéiques, capacité de protection cardiologique et hépatique, agent anti-inflammatoire ainsi que des activités antimicrobiennes et antifongiques (Bellik et al., 2019; Hashim et al., 2017; Yagi et al., 2020). Gbogbo et al. (2006), ont également évalué l'activité antifongique de l'huile essentielle de *C. schoenanthus* vis-à-vis de cinque espèces fongiques (*Alternaria alternata*, *Aspergillus flavus*, *Bipolaris maydis*, *Fusarium oxysporum*, et *Nigrospora oryzae*). Les résultats obtenus montrent qu'à la concentration de 1,14 µL/mL, l'inhibition de la croissance des souches a atteint les 100%, à l'exception de la souche *Nigrospora oryzae*, avec un pourcentage d'inhibition de l'ordre de 96%.

L'activité antifongique d'huile essentielle de *C. schoenanthus* a été évaluée sur un isolat pathogène de *Alternaria* sp. obtenu à partir des feuilles malades de tomates collectées dans des champs paysans dans la localité de Leguema, située à 17 km de la ville de Bobo Dioulasso dans la province du Houet (Burkina Faso). L'huile essentielle stoppe la croissance mycélienne de l'isolat pour des doses allant de 5% à 100%. Dans cette expérimentation et pour préparer les différentes concentrations (1%, 5%, 10% et 50%) d'huile essentielle, le diméthylsulfoxyde (DMSO) a été utilisé comme solvant pour la dilution des huiles essentielles.

1.3.4 *Cymbopogon nardus* (Citronnelle de Ceylan)

1.3.4.1 Présentation de la plante et description botanique

C*ymbopogon nardus,* graminée originaire d'Inde et de Ceylan (Figure 10), s'appelle aussi verveine des Indes en raison de la forte odeur citronnée de ses feuilles. Elle est persistante et forme des touffes denses de tiges lisses. Les longues feuilles odorantes sont linéaires, raides et étroites. Les feuilles sont coupées à la faucille, puis assemblées en fagots pour être acheminées vers la distillerie. La plante a une durée de vie de six à neuf ans avec un rendement en essence optimale dans les trois à cinq ans de vie de cette plante à la fragrance fraiche de note citralée (Baudoux, 2017).

Figure 10 : Une vue complète de la plante de *Cymbopogon nardus* (Saputra et al., 2020).

1.3.4.2 Huile essentielle de *Cymbopogon nardus*

Composition chimique de l'huile essentielle *Cymbopogon nardus*

L'une des premières études menées sur la composition chimique de *Cymbopogon nardus*, rapportée par Heiba & Rizk (1986), a montré que les principaux constituants de *C. nardus* sont : camphène, caryophyllène, cymène, limonène, myrcène, ocimène, phellandrène, pinène, terpinène, terpinolène, thuyène, bornéol, citronellol, élémol, géraniol, linalol, menthane, nérol, pipéritol, alcool propylique, terpinéol, 4-terpinéol, citral, citronellal, méthyl-hepténone, pipéritone, acétate de citronellyle, acétate de géranyle, géranylbutyrate, chavicol, eugénol, méthyl-eugénol, méthyl-isoeugénol, acide citronellique, oxyde de caryophyllène.

L'huile volatile distillée à la vapeur obtenue à partir d'herbe partiellement séchée de *C. nardus* (Nilgiri Hills, Ooty, Inde) contenait 35 composants, dont 29 constituaient 92,7% du total des volatils qui ont été complètement identifiés, tandis que six constituants (7,3%) n'ont été que partiellement identifiés. L'huile contenait 16 monoterpènes (73,3%), cinq hydrocarbures (8,9%), deux aldéhydes (30,0%), huit alcools (40,4%) et un hydrocarbure phénolique (0,5%). Le citronellal (29,7%) était le principal composant identifié, suivi du géraniol (24,2%), du terpinéol (9,2 %), de l'hydrate de cis-sabinène (3,8%), du myrcène (2,9%), du bornéol (2,5%), du nérol (1,5%). Neuf sesquiterpènes (11,5%), dont six hydrocarbures (5,0 %) et trois alcools (6,5 %), ont également été identifiés. Le (E)-nérolidol (4,8%) était le sesquiterpène prédominant, suivi du caryophyllène (2,2%) et du germacren-4-ol (1,5%). Quatre constituants non terpéniques, représentant 1,4% de l'échantillon, ont été détectés, le principal constituant aliphatique étant le 3,3,5-triméthyl-1,5-heptadiène (0,7%). Parmi les six composants partiellement identifiés, la moitié d'entre eux était des monoterpènes. Ces composants comprenaient l'ester bis (2-éthyl hexyl)-1,2-benzène dicarboxylique (2,0%) et un dérivé de l'acide benzène dicarboxylique (1,7%) (Mahalwal & Ali, 2003).

Une comparaison de la composition chimique qualitative et/ou quantitative d'huiles essentielles de *C. nardus* obtenues par hydrodistillation à partir de feuilles du Bénin et du Congo a été réalisée par Abena et al. (2007). Les constituants les plus importants étaient les monoterpènes (plus de 91% dans l'huile essentielle du Congo et 86% dans celle du Bénin) représentés par le citronellal et le géraniol (respectivement 41,3 et 23% dans l'huile essentielle du Bénin contre 37,5 et 29,4% dans celle du Congo). Ces monoterpènes étaient associés à leurs acétates (4,4 et 1,7% dans l'essence du Bénin contre 1,5 et 1,8% pour l'échantillon du Congo). Les composés

oxygénés étaient également abondants et avoisinaient 90% des essences totales. Les proportions d'aldéhydes étaient élevées (40% en moyenne). Quelques sesquiterpènes étaient présents dans ces préparations, représentés par le farnésène (1,5%) et l'élémol (4,8%) dans l'espèce *C. nardus* du Bénin et par l'élémène (1,5%), le trans-caryophyllène (2,5%) et le cadinène pour l'huile essentielle congolaise. Les hydrocarbures (myrcène et limonène exclusivement) ne représentaient que 3%**.** Récemment, une étude réalisée par Saputra et al., (2020) a été menée sur l'huile essentielle obtenue par distillation de feuilles de *C. nardus* séchées à l'air pendant les 24 heures précédentes. L'analyse a révélé que les feuilles de *C. nardus* avaient un total de 29 composés actifs parmi lesquels ont été identifiés : carbamate d'ammonium (18,26%), carbinol (13,57%), néophytadiène (11,65%), trans-géraniol (6,92%), phénol-méthoxy (6. 15%), norolean-12-ène (4,93%), benzofurane (3,9%), guaiacol (3,23%), hexadecene-phytol (3,1%), beta-citronellol (2,69%), trans-caryophyllène (2,61%), alpha-humulène (2,45%) et valérol (2,38%).

Activités biologiques des huiles essentielles de *Cymbopogon nardus*

L'huile essentielle de *Cymbopogon nardus*, dont la composition chimique est variée, possède de nombreuses propriétés. Elle possède des bioactivités potentielles de grande importance pharmaceutique et médicinale. Ces bioactivités sont notamment une activité acaricide, anti-inflammatoire, anticancéreuse, antioxydante et anti-insectes (da Silva et al., 2020 ; Kumoro et al., 2021) ainsi que des activités antimicrobienne et antifongique très fortes. En effet, l'essai de contact utilisant l'huile essentielle et le citronellal a montré une inhibition de la croissance de 3 espèces fongiques. Une concentration de 1,47 mg/mL n'a permis de réduire la croissance d'*Aspergillus* sp. qu'à 90% après 14 jours d'exposition. Pour l'essai de fumigation, 0,05, 0,11 et 0,23 mg/mL d'huile essentielle et de citronellal ont considérablement affecté la croissance de *P. grisea, Aspergillus* spp. et *Colletotrichum musae*. Des effets néfastes sur la sporulation et la germination des 3 champignons ont été observés, et une inhibition complète a été observée à 0,15 mg/mL avec l'huile et le citronellal (Aguiar et al., 2014a)**.**

Ji et al., (2019) ont montré que l'huile essentielle de *Cymbopogon nardus* avait une activité inhibitrice sur *Penicillium corylophilum*, avec une concentration minimale inhibitrice (CMI) de 0,3125 µL/mL. Un échantillon d'huile essentielle de *Cymbopogon nardus* ainsi que son composé principal, le citronellal (36,6%), ont été testés pour leur activité antifongique contre *Pyricularia (Magnaporthe) grisea*, *Aspergillus* spp. et *Colletotrichum musae*. La CMI était de

50 à 100 ppm pour l'huile essentielle et de 25 à 50 ppm pour le citronellal.*Eucalyptus camaldulensis*

1.3.4.3 Présentation de la plante et description botanique

E. camaldulensis est une plante de la famille des Myrtaceae. Généralement appelée "gomme de la rivière rouge", *E. camaldulensis* est un arbre du genre *Eucalyptus* (Figure 11). Les espèces du genre *Eucalyptus* sont cultivées et largement étudiées depuis de nombreuses années dans le monde entier (Luqman et al., 2008). Ce sont généralement de grands arbres qui contiennent une huile essentielle volatile très importante commercialement, notamment pour l'industrie de la parfumerie et de la pharmacie (Batish et al., 2006). *E. camaldulensis* est parmi les espèces les plus importantes du genre. L'arbre est d'environ 24 - 40 mètres de haut avec un tronc robuste, l'écorce est lisse, gris blanc ou chamois et ses feuilles sont persistantes (Boily & Puyvelde, 1986 ; Rats, 2011)**.** C'est une espèce plantée dans de nombreuses parties du monde, dont le Nigéria, mais elle est originaire d'Autriche où elle est répandue surtout au bord des cours d'eau navigables.

Figure 11 : Une vue complète de la plante *Eucalypthus camaldulensis* (https://www.jardineriaon.com/eucalyptus-camaldulensis.htm).

1.3.4.4 Huile essentielle de *Eucalyptus camaldulensis*

Composition de l'huile essentielle *Eucalyptus camaldulensis*

L'analyse chimique de l'huile essentielle de *E. camaldulensis* par Da Cruz et al. (2001), extraite par hydrodistillation, a révélé le 1,8-cinéole (eucalyptol) (43%), l'alpha-pinène (5,5%), le bêta-pinène (3,4%), le p-cymène (5,2%),

Les principaux constituants d'huile essentielle des feuilles de *Eucalyptus camaldulensis* cultivé à Mersin en Turquie étaient le p-cymène (42,1%), l'eucalyptol (14,1%), l'α-pinène (12,7%) et l'α-terpinol (10,7%). Les principaux constituants de l'huile provient des fruits étaient l'eucalyptol (34,5%), le p-cymène (30,0%), l'α-terpinol (15,1%) et l'α-pinène (9,0%). Les résultats montrent que les deux types d'huiles sont riches en termes d'hydrocarbures monoterpéniques et de monoterpènes oxygénés (Dogan et al., 2017).

Une étude effectuée par Ndiaye et al., (2018) montre que les principaux composants des huiles des feuilles d'*E. camaldulensis* récoltées dans trois zones agro-écologiques du Sénégal étaient, pour les huiles essentielles venant de Saint-Louis, très riches en monoterpènes parmi lesquels les molécules oxygénées (principalement le 1,8-cinéole) étaient les plus abondantes (de 74,6 à 78,7 %) suivies des monoterpènes hydrocarbonés (16,7 à 17,8 %). Les huiles essentielles *d'E. camaldulensis* de Dakar et de Kaolack étaient dominées par les monoterpènes hydrocarbonés (38,6–53,1% et 58,4–63,2% respectivement) et par les sesquiterpènes oxygénés (26,2–41,0%) dans les huiles essentielles de Dakar. Dans les échantillons de Saint-Louis, le 1,8-cinéole et le limonène étaient les principaux constituants avec des teneurs respectivement de 69,3–73,0% et 12,4–13,5%. Les huiles essentielles d'eucalyptus de cette région se réfèrent plus étroitement aux données rapportées pour les produits commerciaux. Les principaux composants des huiles d'*E. camaldulensis* de Kaolack étaient le p-cymène (28,4 à 32,6 %), le 1,8-cinéole (16,8 à 20,0 %), le α-pinène (8,7 à 11,6 %) et le limonène (7,8 à 8,3 %). Les huiles de Dakar contenaient du β-pinène (25,3–34,4%) et du α-eudesmol (12,3–20,6%) comme constituants majeurs.

Activités biologiques des huiles essentielles d'*Eucalyptus camaldulensis*

Les feuilles d'*Eucalyptus camaldulensis* sont utilisées en médecine traditionnelle comme antiseptique et pour le traitement de la toux et du rhume, des maux de gorge, de la diarrhée, de la dysenterie et d'autres infections (Ashraf et al., 2015).

Un large éventail d'activités biologiques ont également été évaluées, telles que des activités larvicides, cytotoxiques, antioxydantes, antidiabétiques, anti-inflammatoires, antimicrobiennes et antifongiques (Aleksic Sabo & Knezevic, 2019 ; Ashraf et al., 2015 ; Üstüner et al., 2018).
Les huiles essentielles de feuilles et de fruits de *E. camaldulensis* ont inhibé de manière significative la croissance des bactéries Gram-positives (*Staphylococcus aureus, Streptococcus sp*. et *Bacillus subtilis*) et Gram-négatives (*Escherichia coli*). Les huiles ont également montré une activité fongicide contre *Candida tropicalis* et *C. globrata*. Les huiles essentielles de feuilles ont montré une plus importante activité que les huiles essentielles de fruits, probablement en raison de la concentration plus élevée de p-cymène dans les feuilles (Dogan et al., 2017)**.**
Une étude de Gakuubi et al., (2017), suggérant que l'huile essentielle d'*E. camaldulensis* pouvait être une alternative possible aux fongicides synthétiques, a confirmé les propriétés fongicides d'une huile essentielle de *E. camaldulensis* et son utilisation potentielle dans la gestion des *Fusarium* spp. L'huile essentielle de *Eucalyptus camaldulensis* testée contre cinq *Fusarium* spp. Communément associés au maïs a permis une inhibition complète de la croissance mycélienne de tous les agents pathogènes testés à une concentration de 7-8 µL/mL après cinq jours d'incubation. La concentration minimale inhibitrice et la concentration minimale fongicide de l'huile essentielle sur les champignons testés étaient respectivement de 7-8 µL/mL et de 8-10 µL/mL.

1.4. Les souches fongiques mycotoxinogènes utilisées dans l'étude

Le choix des souches fongiques pour cette étude s'est porté sur deux souches appartenant au genre *Aspergillus*, qui regroupe plus de 200 espèces. Il est caractérisé par une structure particulière ressemblant à l'aspergillum, un instrument utilisé pour asperger l'eau bénite, et qui a inspiré à Micheli, un prêtre catholique, ce nom en 1729 (Amaike & Keller, 2011; Klich, 2009). La première est une souche *A. flavus* (Figure 12), gracieusement fournie par l'unité de recherche Toxalim, Toulouse, France, dont les colonies se caractérisent par une couleur vert-jaunâtre à vert-olive, et relativement planes. L'exsudat est discret. Les revers sont incolores à rosâtres sur milieu Czapek et jaunâtres sur milieu MEA. Certaines colonies peuvent présenter des sclérotes bruns ou noirs et de taille variable (>400 µm). Les conidiophores sont longs et d'une taille généralement inférieure à 1 mm. Leur paroi est épaisse et très rugueuse. La vésicule est globuleuse ou sub-globuleuse (10 à 65 µm de diamètre). Les têtes sont radiées et majoritairement bisériées (deux rangées de stérigmates). Les conidies sont lisses ou légèrement rugueuses (Hedayati et al., 2007).

A. flavus produit des aflatoxines et principalement l'aflatoxine B1. L'AFB1 est classée comme cancérigène par le IARC (Centre International de Recherche sur le Cancer) (groupe 1). C'est un puissant hépato-carcinogène et induit des tumeurs principalement dans le foie, mais aussi dans les reins, les poumons et le colon chez l'homme et les animaux (Amaike & Keller, 2011). Au niveau économique, les aflatoxines affectent de nombreuses cultures agricoles telles que le riz, le maïs, le blé, l'arachide et le piment. La contamination de ces cultures par les aflatoxines avant et après la récolte est fréquente et entraîne chaque année d'importantes pertes économiques (Tian & Chun, 2017).

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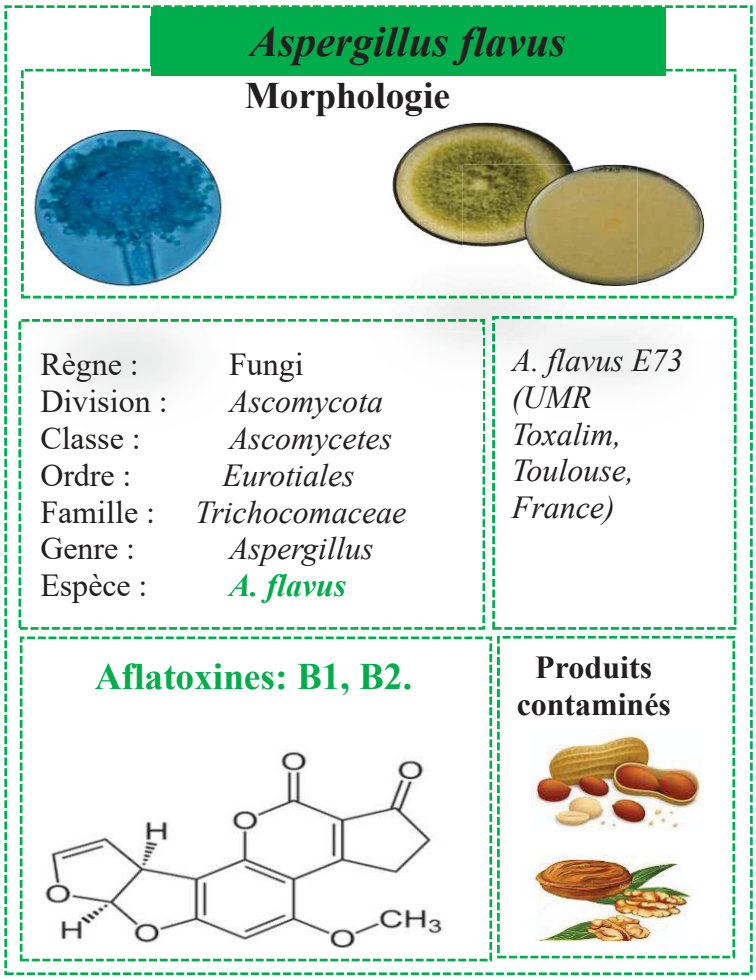

Figure 12 : Présentation de *Aspergillus flavus* et de ses caractéristiques.

La deuxième est *A. carbonarius*, provenant de la collection l'unité de recherche QualiSud, Montpellier, France (Figure 13). Cette souche appartenient au groupe *Aspergillus* section Nigri (black *Aspergilli*). *A. carbonarius* est un champignon filamenteux noir qui colonise notamment le cacao et le café, les raisins ainsi que les fruits secs (Battilani et al., 2006; Malir et al., 2016; Veras et al., 2021). Son aspect macroscopique ne permet pas sa différenciation des autres black *Aspergilli*, en raison des ressemblances morphologiques macroscopiques entre les champignons noirs. Néanmoins, au niveau microscopique, *A. carbonarius* a une structure assez particulière : c'est un champignon bisérié ayant des conidiophores lisses de 2 à 3 µm de longueur, des vésicules sphériques de diamètre 60 à 90 µm, les métules sont de 12 à 18 µm et de très larges conidies de 6 à 8 µm de diamètre, noires et très rugueuses, voire pointues. En plus de ces caractéristiques morphologiques, *A. carbonarius* est le seul à pousser dans une gamme de température allant de 9 °C jusqu'à 36 °C (Samson et al., 2007)

L’IARC a classé l'OTA comme agent cancérigène possible pour l'homme (groupe 2B) en 1993, sur la base d'un grand nombre de preuves de sa cancérogénicité découvertes dans plusieurs études animales (Malir et al., 2016).

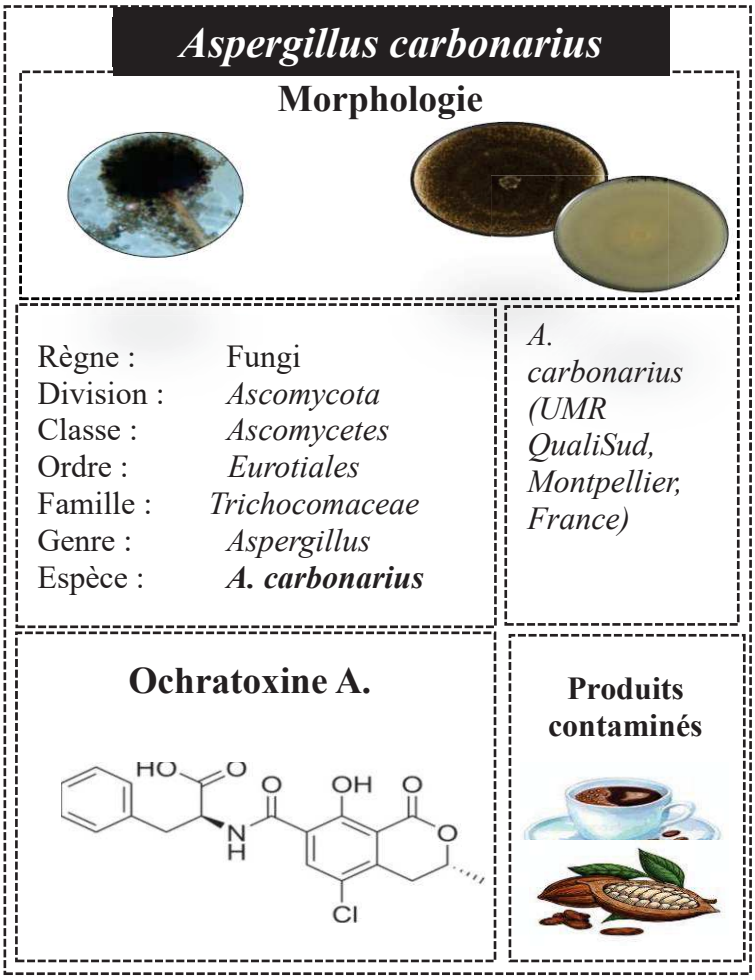

Figure 13 : Présentation de *Aspergillus carbonarius* et de ses caractéristiques.

La troisième souche étudiée appartient au genre *Fusarium*. *F. verticillioides* BRFM 2251, qui été gracieusement fournie par l’unité de recherche CIRM, Université d'Aix-Marseille, Marseille, France (Figure 14), est un champignon phytopathogène et mycotoxinogène, causant d’importants dégâts sur les céréales. Cette souche a prouvé sa capacité à produire d’importantes quantités de fumonisines et spécialement la fumonisine B1. L’IARC a classé la FB1 comme agent cancérigène possible pour l'homme (groupe 2B). *In vitro* et sur milieu nutritif optimal, les colonies de *F. verticillioides* se développent progressivement et forment des amas mycéliens très denses et aux bordures relativement lobées. La couleur du mycélium est d’abord blanche puis présente des nuances de rose et de violet clair. Après un développement plus important,

des pigments sont produits de manière radiale avec des couleurs sombres du violet au bleu/noir. Les micro-conidies apparaissent portées par le mycélium aérien sous forme de longues chaînes et ont une forme ovoïde avec une base légèrement aplatie (4-10 µm). Elles sont produites en très grande quantité et avec des tailles assez variables même dans les mêmes conditions. La plupart des souches ne produisent que très peu de macro-conidies (Leslie & Summerell, 2008).

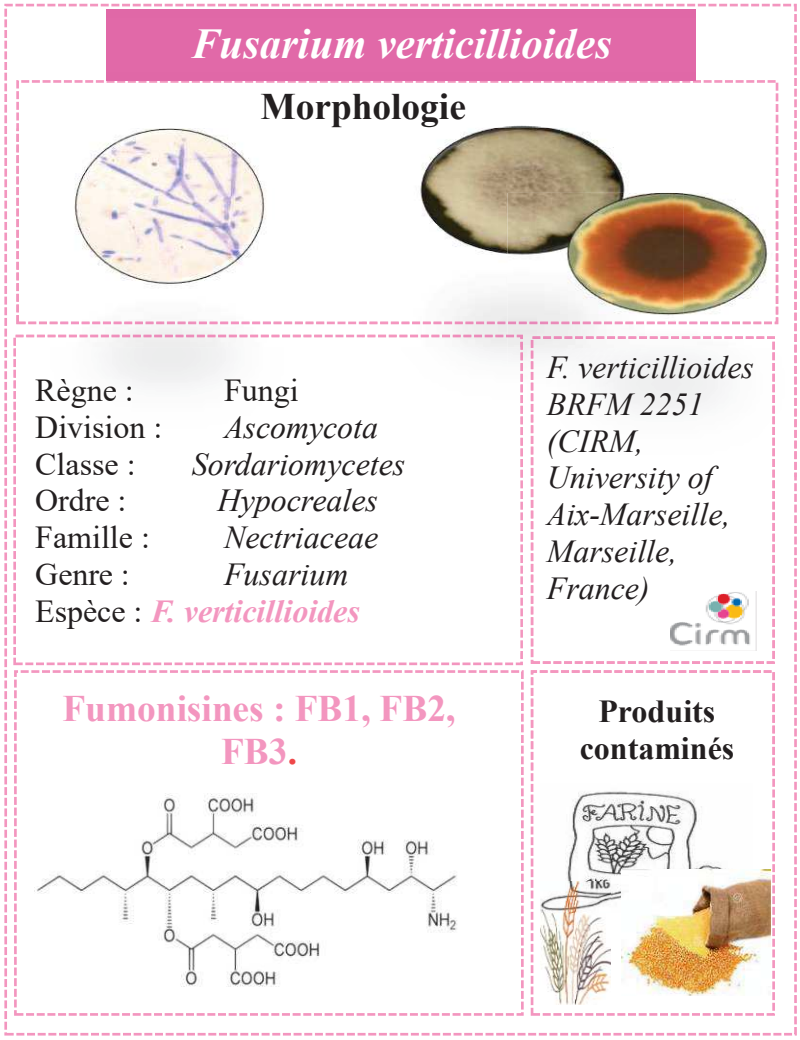

Figure 14 : Présentation de *Fusarium verticillioides* et de ses caractéristiques.

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Résultats Expérimentaux

2 Résultats expérimentaux

Les mycotoxines sont des métabolites secondaires toxiques produits par certaines espèces de champignons filamenteux, qui apparaissent naturellement dans les produits alimentaires dans des conditions favorables de température et d'humidité, entre autres facteurs (Gonçalves et al., 2018). Les souches fongiques mycotoxinogènes sont connues pour leur capacité à coloniser différents types d'aliments tels que les céréales, les épices, les légumes, ou les fruits. Les mycotoxines sont l'une des causes les plus importantes des pertes alimentaires et sont devenues un problème récurrent de sécurité alimentaire (Ayelign & De Saeger, 2020). Les mycotoxines peuvent en outre avoir des effets aigus et chroniques variés sur les humains et les animaux (Atanasova-Penichon et al., 2016; Ine van der Fels-Klerx et al., 2018; Veprikova et al., 2015). Elles peuvent en effet présenter des effets génotoxiques, cancérigènes et immunosuppresseurs (Dammak et al., 2019). La consommation d'aliments contaminés par des mycotoxines a été associée à plusieurs cas d'empoisonnement humain ou de mycotoxicose, entraînant parfois la mort (Bluma et al., 2008).

Associée à de bonnes pratiques agricoles, l'utilisation des fongicides est un facteur clé dans les stratégies de gestion intégrée visant à lutter contre ce problème économique et de santé publique. Leur utilisation répétée pendant des décennies a cependant perturbé les systèmes biologiques naturels et entraîné le développement de résistances fongiques et les résidus de ces fongicides posent un problème sanitaire pour la santé humaine (Chen et al., 2019). De ce fait, l'utilisation de fongicides synthétiques devient de plus en plus restrictive en raison de leur cancérogénicité, de leur toxicité résiduelle élevée et aigue et de leur longue période de dégradation et donc leur effets néfastes sur l'environnement (Dammak et al., 2019). Ainsi, le règlement européen REACH et les plans français Ecophyto ont été mis en place pour diminuer l'utilisation des fongicides de synthèse dans les prochaines années. Il existe donc un besoin important d'alternatives biologiques et non toxiques, efficaces pour prévenir la croissance des moisissures et leur production de mycotoxines.

Ces dernières années, les produits de biocontrôle à base de plantes ont fait l'objet d'une attention accrue et sont considérés comme capables de répondre à ces exigences (Rahmat et al., 2014).

2.1 Activité antifongique et anti-mycotoxinogène de l'Antoferine®

Introduction

Afin de trouver un équilibre entre la prévention des pertes alimentaires et la réduction des risques pour les consommateurs, l'entreprise Antofénol explore de nouvelles technologies éco-responsables permettant d'extraire à partir des déchets de la vigne des composés antifongiques comme l'Antoferine®. Des études ont prouvé son efficacité dans l'inhibition de la croissance de nombreuses souches fongiques mais ces études se sont alors limitées aux effets fongicides ou fongistatiques de l'Antoferine® et son effet sur la contamination par les mycotoxines n'a pas encore été examiné. À cet égard, l'objectif de cette étude a donc été d'étudier l'effet de l'Antoferine® sur trois souches fongiques mycotoxinogènes : *Aspergillus flavus*, *Aspergillus carbonarius* et *Fusarium verticillioides*.

De plus, et pour mieux comprendre les relations composition/bioactivité, une étude sur l'effet antifongique de deux de ses principaux composants bioactifs (Trans-resvératrol et Trans-Ɛ-viniférine) a été réalisée.

Article soumis dans la revue Mycotoxin Research: Antifungal activity of Antoferine®, a commercial biocontrol product against three mycotoxinogenic fungi

Antifungal activity of Antoferine®, a commercial biocontrol product against three mycotoxinogenic fungi

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Abstract

The humans are regularly exposed to mycotoxins, mainly through food intake of plant and animal origin. They are considered a real food safety issue, due to their adverse health and socio-economic impacts. In order to limit the use of chemical fungicides to control the mycotoxic risk, alternative environmentally friendly solutions are increasingly studied. Among the most promising strategies is the use of bioactive plant extracts such as phenolic compounds which are reported to be effective in the inhibition of phytopathogenic and/or mycotoxigenic fungi.

Antoferine® showed a dose-dependent activity on the growth and toxin production, and to a lesser extent on the production of spores, of *A. flavus* and *F. verticillioides*. Its effect on the growth of *A. carbonarius* is more contrasted since growth is only slightly impacted and toxin production is stimulated. Antoferine® has also shown potential for detoxification of aflatoxin B1 produced by *A. flavus*.

Antoferine® seems to be a promising solution for the biocontrol of certain toxigenic fungi and their associated mycotoxins. An extended study to other mycotoxigenic strains and the deciphering of the mechanisms of action are now to be considered.

Keywords:

Antoferine®, Phenolic compounds, Fungi, Mycotoxin.

Introduction

Phytopathogenic fungi are responsible for nearly 25 % of all crop diseases and may have a high impact on crops, affecting them during cultivation, post-harvest or storage (Eskola et al., 2020). From an economic concern, they can cause high yield losses by damaging host plant and its production. Moreover, some of these fungi are known to produce mycotoxins which constitute a health risk for human or animal consumers. Mycotoxins are secondary metabolites produced by fungi in plant-based food. They are considered a real issue in food safety, due to their acute and chronic toxic effects on animals and humans (Neme & Mohammed, 2017; Ostry et al., 2017). Filamentous fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* are most related to the production of these toxins (Marin et al., 2013; Tola & Kebede, 2016). Out of a large list of compounds identified as mycotoxins (>400), there are only a few (30) that receive the most attention, and are responsible for significant health risks and economic losses (Medina et al., 2017). The most important mycotoxins are aflatoxins (AFs) (represented mainly by aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1)), ochratoxins (OTs) (represented mainly by ochratoxin A (OTA)), fumonisins B (FBs) (represented mainly by fumonisins B1 (FB1), B2 (FB2), and B3 (FB3)) (Haque et al., 2020; Tao et al., 2018). control of mycotoxin contamination still relies mainly on the use of chemical fungicides, but the development of drug resistance in many fungal pathogens, as well as growing public concern about the health and environmental effects of fungicides, has led to the search for new alternatives that are both beneficial to public health and safe for the environment (Mari et al., 2016). Among these alternatives, plant extracts are attracting growing interest because they are generally considered to be safer for the environment (Yang et al., 2015). Indeed, plants produce a wide variety of metabolites which serve as defense compounds for their own protection against other plants, parasites and microorganisms, such as phenolic compound (Kalli et al., 2018). known for their antioxidant, anti-inflammatory and antimicrobial activities (Crespo-Sempere et al., 2016; Dai & Mumper, 2010; Jesus et al., 2019). Among them, stilbenes are phenolic compounds of considerable importance because they help the plant to defend itself against attacks from pathogenic microorganisms by acting as phytoalexins (Zwingelstein et al., 2019). Thus, stilbenes have shown significant antifungal activity (Fotso et al., 2020). They are found in many plants and in particular in the grapevine (*Vitis vinifera*) and the stilbenes resulting from the waste of the grapevine have also proved their efficacy against *Plasmopara viticola* and other grape pathogens (Gabaston et al., 2017). Since the production of wine

generates a significant source of waste rich in stilbene, in particular between 1.4 and 2 tons per hectare and per year of branches, this could be an interesting source of bioactive compounds (Sánchez-Gómez et al., 2017). It is in this context that the company Antofénol has developed an innovative antifungal solution of natural origin to protect fruits and vegetables in post-harvest operations. Antoferine® is a natural vine extract obtained through an eco-responsible solvent-free extraction process. Numerous studies have been performed that have proven its efficacy in inhibiting the growth of many fungal strains (Rolet and Fabre 2016). However, until now, these studies have been limited to the fungicidal or fungistatic effects of Antoferine® and its effect on the production of mycotoxins has not yet been studied.

Thus, this study aimed to examine the effect of Antoferine® and its main stilbene components against three mycotoxinogenic fungi, *A. flavus*, *A. carbonarius* and *F. verticillioides,* with regard to their growth and sporulation. Further, particular attention was paid to the effect of Antoferine® on their production of mycotoxins, AFB1, OTA and FB1 respectively.

Materials and methods

Fungal strains

Toxigenic filamentous fungal strain *A. flavus* E73 (UMR Toxalim, Toulouse, France), *A. carbonarius* (property of UMR QualiSud, Montpellier, France) and *F. verticillioides* BRFM 2251 (CIRM, University of Aix-Marseille, Marseille, France) were chosen because of their strong ability to produce their respective mycotoxins (Ahmed et al., 2015a; Campos-Avelar et al., 2020; Pellan et al., 2020).

Preparation of spore suspensions

All fungi were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) under paraffin oil at 4 °C and actively grown on PDA at 25 °C for 7 days for spore production. Spore suspensions were prepared by adding 10 mL of a sterile distilled water to the plate then by scrapping the surface of the cultures with a sterile spreader. The spore suspensions were collected and theirs spores concentration were determined using Thoma cell counting chamber. Spore concentrations were then adjusted to 10^6 spores/mL.

Commercial biocontrol product

Commercial biological control product used in this study is Antoferine® which is a natural vine extract that contains polyphenols, produced by Antofénol company (Plestan, France).

Inhibition of fungal growth

The antifungal activity of Antoferine® were determined by measuring the fungal colonies area exposed to this product. Antoferine® was mixed with PDA medium up to final concentrations (0, 1, 2, 3, 4 ,5,10, 20 and 30 g/L). Antoferine®-free medium was used as control. All the treatments and control were inoculated with 5 µL of a spore suspension adjusted to 10^6 spores/mL of each fungal strain in the center of the plate and incubated in the dark at 25 °C, for 12 days. Every three days, Petri dish were photographed, and the colony area of each fungus

were measured in cm^2 using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018).
All assays were carried out in triplicates for each condition.

Quantification of fumonisin B1, ochratoxin A and aflatoxin B1

Mycotoxin extractions

The concentration of mycotoxins in fungal cultures, from each culture conditions as well as the control, was determined after 3, 6, 9 and 12 days of cultivation. Both fungal biomass and culture medium were used for mycotoxins analysis. Half of the contents of a Petri dish was placed in pot and weighed. For *F. verticillioides* cultures, 50 mL of water/acetic acid (99.5:0.5, v/v/v) were added. A mixture of methanol/formic acid (25:1, v/v) from VWR (Fontenay-Sous-Bois, France) was added for both *Aspergillus* strains cultures, the quantity of solvent used was 25 mL for *A. carbonarius* and 50 mL for *A. flavus.* All samples were homogenized by mechanical agitation using an orbital shaker at 250 rpm for 20 min. Extraction solvent from *F. verticillioides* samples were filtered directly with a CA filter 0.45 µm (Carl Roth GmbH, Karlsruhe, Germany). A total of 500 µL of the mixture from *Aspergilli* samples were evaporate until dryness at 60 °C in SpeedVac vacuum concentrator (Eppendorf® AG, Hamburg, Germany). Residues were taken up in 2 mL of solvent corresponding to the mobile phase used for HPLC analysis, then placed in an ultrasonic bath for 20 minutes and filtered through a 0.45 µm PTFE filter (Dominique Dutscher S.a, Grabels, France).

Mycotoxin analysis

Fumonisin B1 in the samples was quantified by Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan). The operating conditions were: injection volume of 50 µL; HPLC column $C_{18,}$ Phenomenex Kinetex XB (50 mm× 2 mm; 2.6 µm particles), at 50°C, isocratic flow rate of 0.4 mL/min. The mobile phase consists of 0.5% acetic acid in ultra-pure water (A) and 0.5% acetic acid in isopropanol (B) (HPLC MS grade, Sigma, St Louis, MO, USA). The mobile phase gradient program was started at 90% A (0.01 min), 45% A at 1.5 min, 15% A at 3.5 min, 20% A at 4 min, 98% A at 4.01 min and finally 98% A at 11 min.

The mass spectrometer was operated in electrospray positive (ESI+) andnegative (ESI–) ionization mode, and two multiple reaction monitoring (MRM). The transitions for each analyte were monitored for quantification (Q) and qualification (q) (Table 1). All data were analyzed using LabSolution Software (v5.91/2017, Shimadzu, Tokyo, Japan, 2017).Limits of detection or quantification (LOD/LOQ in ng/mL, respectively) FB1 were (0.03/0.1).(Pellan et al., 2020) For OTA the concentration for all experiments was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan). The operating conditions were as follows: injection volume of 100 µL; HPLC column C_{18}, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 35 °C; isocratic flow rate of 1 mL/min (mobile phase: methanol/water/acetic acid, 69:30:1); excitation wavelength of 333 nm and emission wavelength of 460 nm. Detection and quantification limits were established at 0.015 and 0.05 ng/mL respectively. Aflatoxin concentration was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan), after derivatization post column with electrochemical system (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). The operating conditions were as follows: injection volume of 100 µL; HPLC column C_{18}, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40 °C; isocratic flow rate of 0.8 mL/min (mobile phase: water-methanol (55:45; v/v), 119 mg of potassium bromide and 350 µL of nitric acid per L); excitation wavelength of 365 nm and emission wavelength of 435 nm. Detection and quantification limits for Aflatoxin B1 were established at 0.015 and 0.05 ng/mL respectively.(Campos-Avelar et al., 2020) Mycotoxin levels were expressed in ng/cm, specific mycotoxin production.

Table 1. MS/MS parameters for isotope labelled, internal standard (IS) and FB1.

(Q) Quantification; (q) qualification.

	Polarity	MRM Q	EC MRM Q	MRM q	EC MRM q	R^2 calibration curve
U-($^{13}C_{34}$)- FB_1	+	756.3 > 356.5	-47			
FB_1	+	722.35 > 334.5	-43	722.35 > 352.0	-44	0.9982

Effect of Antoferine® on fungal sporulation

The effects of Antoferine® on fungal sporulation were determined using the method described by Vipotnik et al. (2017), with some modifications : mycotoxigenic fungi inoculated on PDA medium with 5 µL of a spore suspension adjusted to 10^6 spores/mL in the center of the plate and incubated in the dark at 25 °C.

Microscopic observations

A. flavus was chosen among the three strains to observe the Antoferine® effect on the spores, hyphae and conidia morphology because of the high susceptibility of AFB1 accumulation to Antoferine®.

Scanning Electron Microscopy

PBS washed spores were fixed with 2.5% glutaraldehyde in PHEM buffer, pH 7.2 for an hour at room temperature, followed by washing in PHEM buffer. Fixed samples were dehydrated using a graded ethanol series (30-100%), followed by 10 minutes in graded Ethanol - Hexamethyldisilazane. And then Hexamethyldisilazane alone. Subsequently, the samples were sputter coated with an approximative 10nm thick gold film and then examined under a scanning electron microscope (Hitachi S4000, at CoMET, Institute des Neurosciences Montpellier, MRI-RIO Imaging, Biocampus, Montpellier, France) using a lens detector with an acceleration voltage of 10KV at calibrated magnifications.

Transmission Electron Microscopy

Spores were immersed in a solution of 2.5% glutaraldehyde in PHEM buffer (1X, pH 7.4) overnight at 4 °C. They were then rinsed in PHEM buffer and post-fixed in a 0.5% osmic acid for 2 h at room temperature in the dark. After two rinses in PHEM buffer, the spores were dehydrated in a graded series of ethanol solutions (30-100%). The spores were embedded in EmBed 812 using an Automated Microwave Tissue Processor for Electronic Microscopy, Leica EM AMW. Thin sections (70 nm; Leica-Reichert Ultracut E) were collected at different levels of each block. These sections were counterstained with uranyl acetate 1.5% in 70% Ethanol and lead citrate (Institute des Neurosciences Montpellier, MRI-RIO Imaging, Biocampus Montpellier France) and observed on transmission electron microscope of brand Jeol model 1200EX2 at 100Kv and the images were captured with a camera of brand SIS Olympus model Quemesa. The point-to-point resolution of the MET is 0.4nm and its resolution on networks is 0.2 nm. The digital camera has a 11 Mpixel CCD sensor (Université de Montpellier, Plateforme Technologique Microscopie Electronique et Analytique).

Mycotoxin detoxification assay

Reagents and solutions for mycotoxin analysis

The mycotoxins standards of AFB1 and FB1 were purchased from Sigma (Sigma, St Louis, MO, USA). Individual stock solutions were prepared in acetonitrile, methanol and acetonitrile/methanol (50:50; v/v) respectively and stored at 4 °C. After the results of the confrontation, it was found that Antoferine® stimulates the production of OTA and that is why it was excluded from the degradation assay.

Mycotoxin detoxification assays

Mixtures of Antoferine® and mycotoxin, AFB1 or FB1, were prepared at concentrations of 0,83 g/L and 16,7 ng/mL or 3,3 µg/mL. A control was prepared for each mycotoxin without Antoferine®. Samples were incubated for 2, 12 or 24 h at 20 or 30 °C. After incubation, 200 µL of the sample were mixed with 800 µL of water/acetic acid (99.5:0.5, v/v) and analyzed in a following the previously described protocols.

Antifungal activity and anti-mycotoxin effect of the main compounds of Antoferine®

The antifungal activity of Trans-resveratrol and Trans-Ɛ-viniferin (5.5 % and 4.7% (w/w) of Antoferine®, respectively), the main compounds of Antoferine®, against three fungi were determined following the protocol described previously. Trans-resveratrol and Trans-Ɛ-viniferin were mixed with 4 mL of PDA medium up to final Trans-resveratrol concentrations of 55.5, 137.5 and 275 mg/L and to final Trans-Ɛ-viniferin concentrations of 235 and 470 mg/L. These concentrations were chosen according to the concentration of Antoferine® effective against mold and the content of Trans-resveratrol and Trans-Ɛ-viniferin in Antoferine® (i.e. 5.5 % and 4.7% (w/w) of Antoferine®, respectively). PDA without Antoferine® compounds was used as control. All modalities were inoculated in duplicate with 10 µL of a spore suspension adjusted to 10^5 spores/mL of each fungal strain in the center of the plate and incubated in the dark at 25 °C, for 4 days. After incubation, colony area of each fungus was measured using the same method described previously

Mycotoxin were quantified according to the protocols described previously, with minor modifications: an appropriate volume of solvents was used for each mycotoxin.

Isolation of fungal RNA and analysis by quantitative real-time PCR (qRT-PCR)

Strain mycelia grown in PDA medium at 25 °C for 5 day and 8 days were peeled off and grinded using GenoGrinder (SpexSamplePrep, NJ, USA) with steel grinding balls. Total RNA purification was performed through NucleoSpin RNA (Macherey-Nagel, GmbH & Co. KG, Germany), following the manufacturer's instructions. RNA concentration was determined by the absorbance at 260 nm. The concentration of all samples was adjusted at 100 ng/µL First-strand cDNA synthesis reaction was carried out using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting cDNA was diluted 1:20 with water. The primers used for real-time PCR were listed in Table 1. Experiments were carried out in 384 wells microplate. Reaction mixtures (final volume of 1.5 µL) consisted of forward and reverse primers (final concentration 600nM each), SYBER GREEN PCR master mix (Applied Biosystems, Scoresby, Vic., Australia) and 0.5 µL of cDNA. Non-template samples with 0.5 µL of distilled water instead of DNA were used as negative controls.

All qPCR runs were done using the LightCycler 480 instrument (Roche Diagnostics, Basel, Switzer- land). Real-time PCRs were performed as follows: a first step was a pre-incubation at

95 °C for 3 min followed by 45 amplification cycles (95 °C for 10 s, annealing at 68 °C or 64 x°C (*aflD* or the others respectively) for 10 s), and a final extending step for 10 s at 72 °C., for melt curve analysis the parameters used are: 95 °C for 5 s, 65 °C for 1 min and up to 97 °C before cooling down to 40 °C. Housekeeping gene β-tubulin was used for data normalization. Relative gene expression ratio of both groups (control and treatments) was determined using the Advanced Relative Quantification LightCycler® 480 Software considering the real-time PCRs efficiencies of the housekeeping gene and the targeted genes (Table 2).

Table 2. List of primers

Target gene	Primer	Sequence (5′ -3′)	Efficiency	References
aflP	*aflP*-F	TGTGTCGAGTGATGTGGGACTAG	2	(Hua et al., 2014)
	aflP- R	GCCACCCAGCTCAACCTACA		
aflD	*aflD* -F	CACCATCACCAACATGCAC	2	(Wilkinson et al., 2007)
	aflD -R	CTGGATCGATGATGAAGGC		
β tubulin	β tubulin-F	GTTGACCCAGCAGATGTTCGA	1.84	(Hua et al., 2014)
	β tubulin-R	TGGAGGTGGAGTTTCCAATGA		
fum 1	*fum 1*-F	ACACCAAAGCCTCTACAGGTGA	2	(Visentin et al., 2012)
	fum 1-R	AGGTATCGGGCACCGCT		
fum 8	*fum 8*-F	AGCACAGACGGCGGAGAAGTT	1.81	(Visentin et al., 2012)
	fum 8-R	TGAGTTGTCGCTCGCTTGTTG		
β tubulin	β tubulin- F	CAGCGTTCCTGAGTTGACCCAACAG	2	(Choi & Shim, 2008)
	β tubulin- R	CTGGACGTTGCGCATCTGATCCTCG		

Statistical analysis

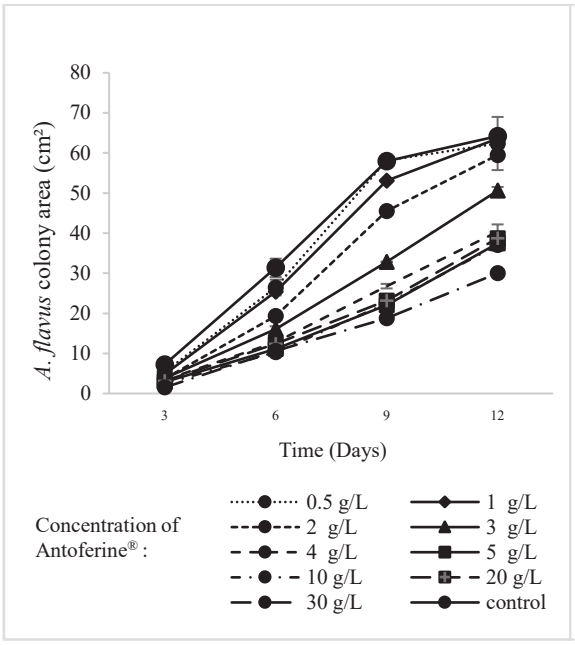
All experiments were carried out in triplicates. All statistical tests were performed using R software (RStudio), version 3.6.3. Data regarding the sporulation, growth, mycotoxin productions, degradation and gene expressions were analyzed through a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$).

Results

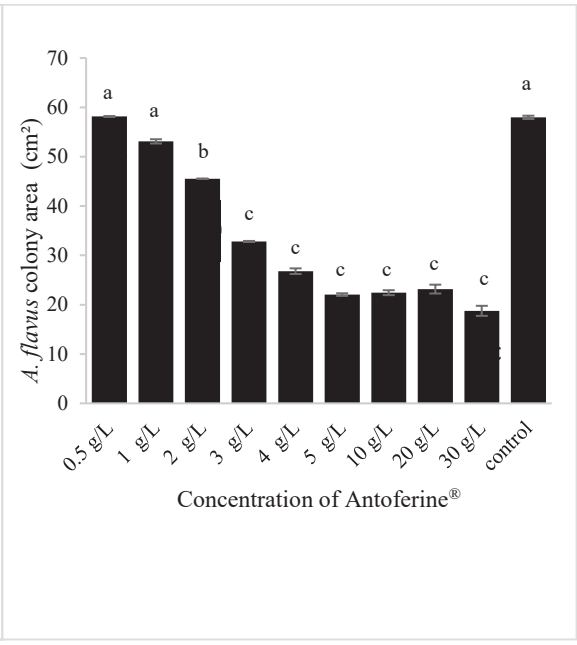
Impact of Antoferine® on fungal growth

The inhibitory effects of Antoferine® on the growth of *A. flavus, A. carbonarius* and *F. verticillioides* were assessed on PDA agar at 25 °C ± 2 for 12 days (see Fig. 1). Antoferine® was shown to affect the fungal growth depending on its concentration and on the strains. The growth kinetics were determined by measuring the area of the fungal colonies for 12 days, starting from day 3 to day 12 after inoculation on PDA medium. The growth in the control cultures gradually increased until day 9, until the strain occupied the entire surface of the Petri dish. As shown in Fig. 1 a, the growth of *A. flavus* was inhibited by Antoferine® in a dose-dependent manner. At day 9 (see Fig. 1 b), the colony surface of the control was 57.9 cm² while it was 18.7 cm² for the concentration of 30 g/L. Statistical analysis at day 9 showed a significative ($P<0.01$) inhibition of the colony growth from 2 g/L. The growth of *A. carbonarius* (see Fig. 1 c) was less impacted but the colony surfaces were significantly different ($P<0.05$) at day 9 in presence of Antoferine® (see Fig. 1 d). At day 9, the colony surface of the control reached 58.1 cm² while it was 49.9 cm² for the concentration of 30 g/L. The growth of *F. verticillioides* (see Fig. 1 e). was significantly ($P<0.01$) impacted by Antoferine® and at day 9 the growth was totally inhibited from the concentration of 10 g/L (see Fig. 1 f). At day 9, the colony surface of the control was 48.1 cm² and 4.6 cm² for *F. verticillioides* grown in PDA supplemented with 5 g/L.

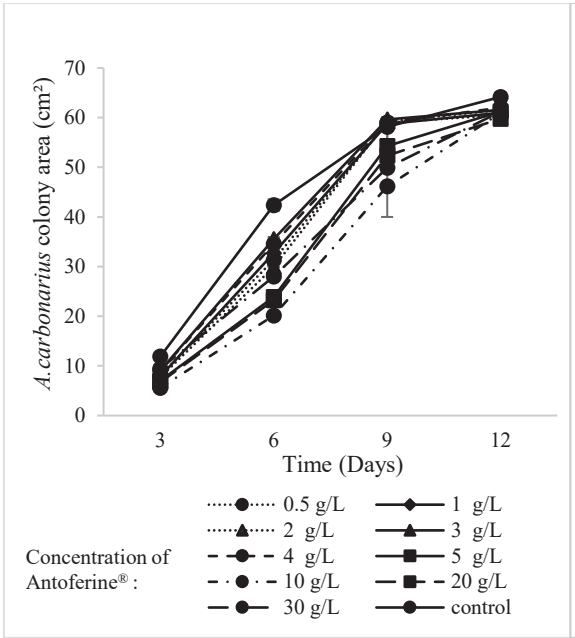
1(a)


A. flavus colony area (cm²)
Time (Days)
Concentration of Antoferine® :
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

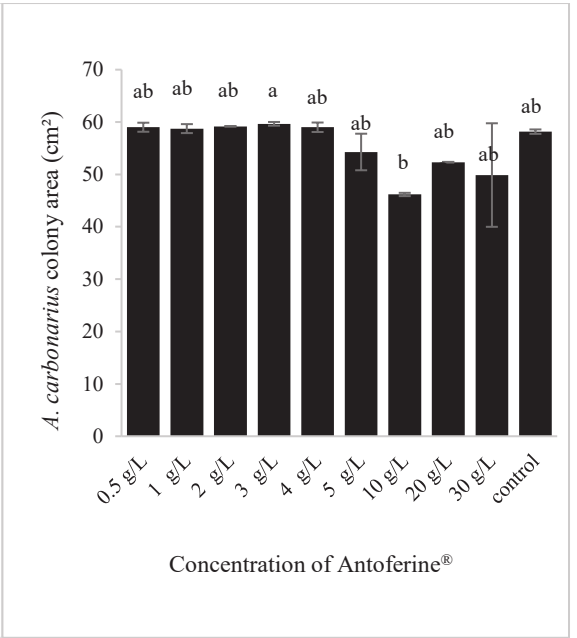
1(b)


A. flavus colony area (cm²)
Concentration of Antoferine®
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

1(c)


A.carbonarius colony area (cm²)
Time (Days)
Concentration of Antoferine® :
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

1(d)


A. carbonarius colony area (cm²)
Concentration of Antoferine®
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

1(e) 1(f)

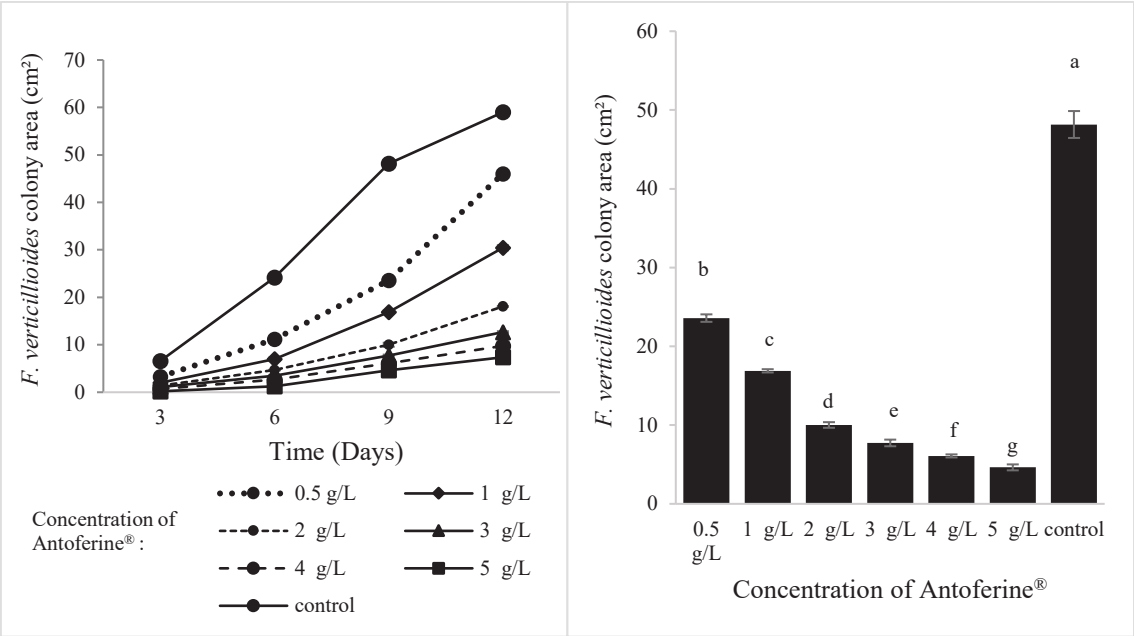


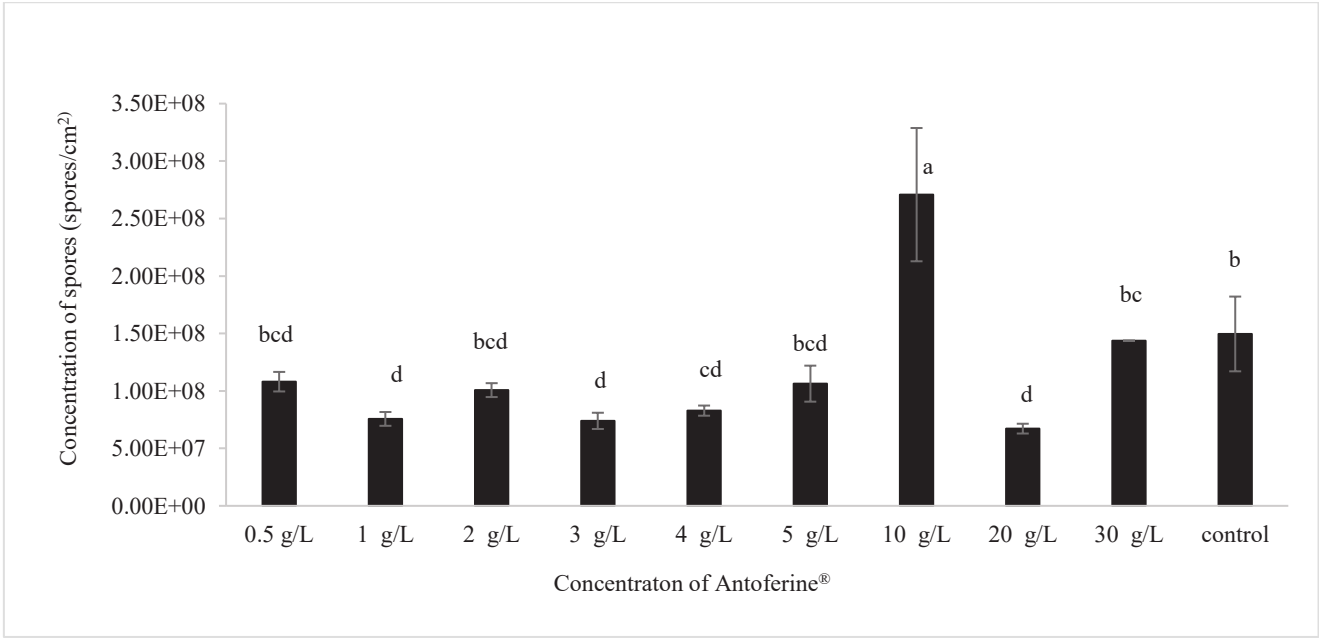
Fig. 1 Effect of different concentrations of Antoferine® on fungal colony surface (cm²) in PDA medium at 25°C. (a) Growth kinetic of *A. flavus* incubated for 12 days. (b) Growth of *A. flavus* on day 9. (c) Growth kinetic of *A. carbonarius* incubated for 12 days. (d) Growth of *A. carbonarius* on day 9. (e) Growth kinetic of *F. verticillioides* incubated for 12 days. (f) Growth of *F. verticillioides* on day 9.

Impact of Antoferine® on spore production

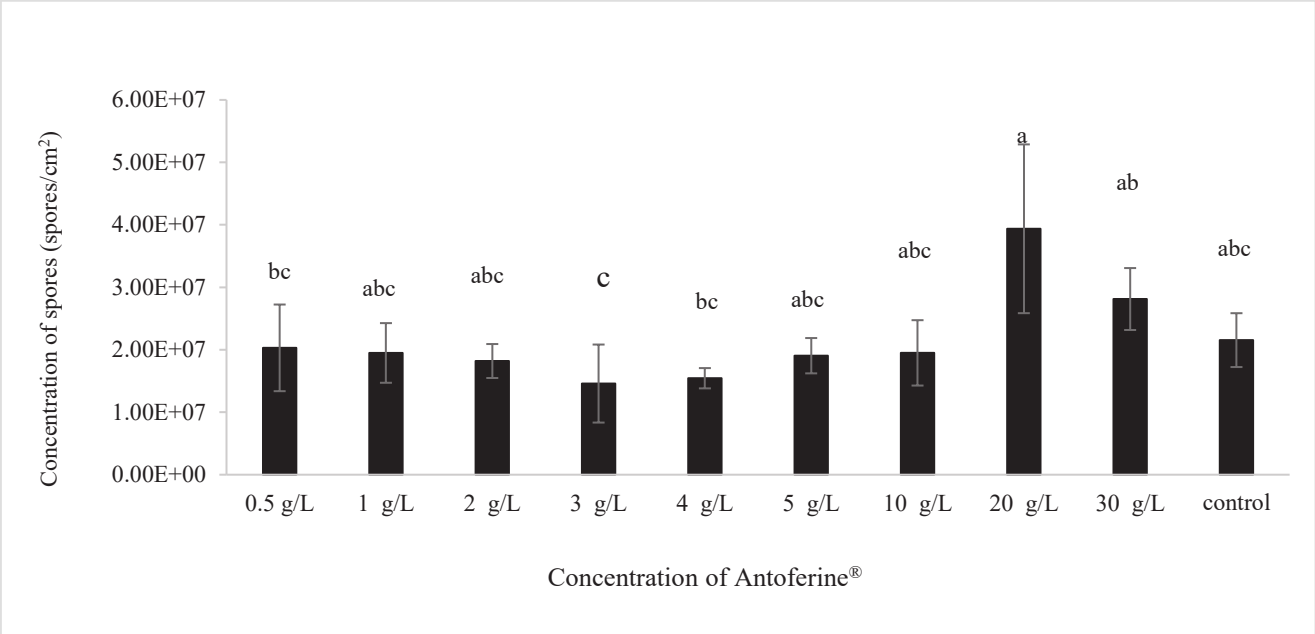
Antoferine® was shown to affect the spore production depending on the strains and the concentration of Antoferine® (see Fig. 2).

On day 5 a significant (P<0.001) decrease in the sporulation of *A. flavus* was observed for 1, 3, 4 and 20 g/L of Antoferine® (see Fig. 2 a) while the concentration of 10 g/L significantly increased the production of spores by *A. flavus.* Concerning *A. carbonarius* (see Fig. 2 b) Antoferine® at the concentrations of 1, 2, 3, 4, and 5 g/L caused a significant decrease (P<0.01) of the spore production and inversely the concentration of 10, 20 and 30 g/L stimulated the production. For *F. verticillioides*, a significant decrease (P<0.05) of the sporulation was only observed for 5 g/L of Antoferine® whereas the concentration of 0.5 g/L increased the production of the spore (see Fig. 2 c).

2(a)



2(b)



2(c)

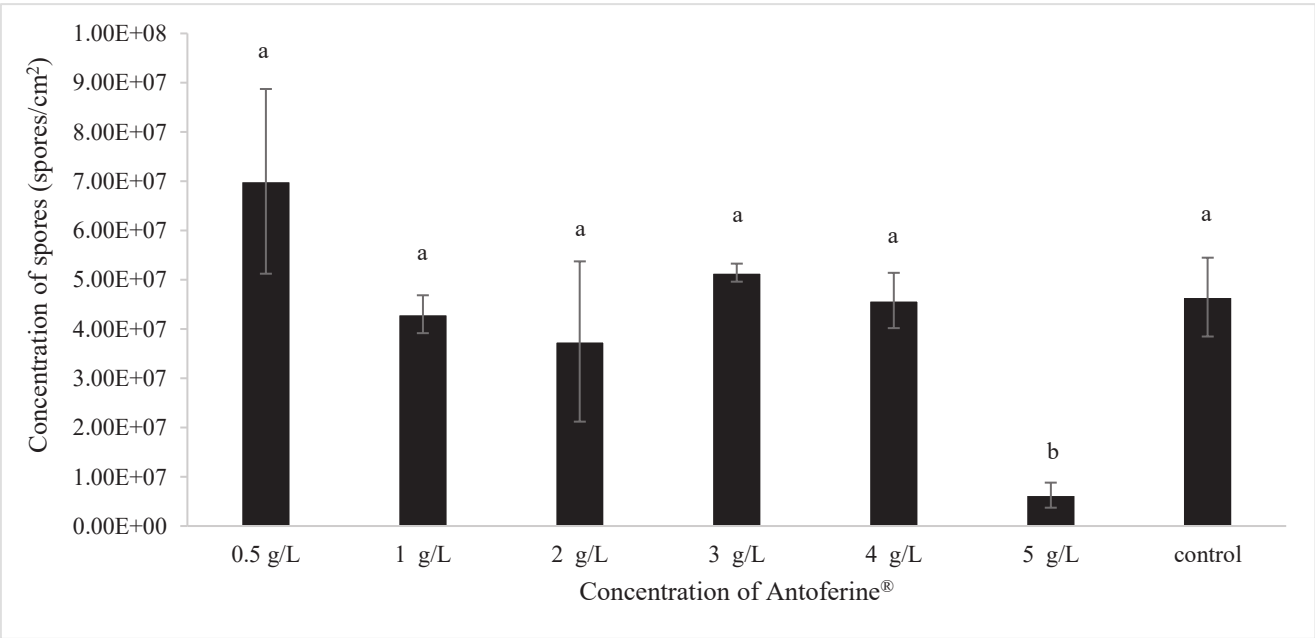


Fig. 2 Effect of different concentrations of Antoferine® on spore production after 5 days in PDA medium at 25 °C. (a) for *A. flavus*. (b) for *A. carbonarius.* (c) for *F. verticillioides*.

Microscopic observations of *A. flavus* grown in presence of Antoferine®

Taking into account the previous results, the choice was made to study the influence of Antoferine® on the cells of *A. flavus* which produces AFB1, classified as carcinogenic by the IARC (International Agency for Research on Cancer).

Scanning electron microscopy (SEM)

When cultivated in presence of Antoferine®, *A. flavus* microscopic structures presented some modifications compared to the control (see Fig. 3). Indeed, SEM on *A. flavus* grown on PDA with 20 g/L of Antoferine® showed that Antoferine® did not affect the size of the conidial heads. However, the conidial heads exhibited rounder heads with shorter phialides. The mycelia also showed modifications of the hyphae. These hyphae became slender, shrank and winding, and lost their linearity with some depressions on the hyphal surface.

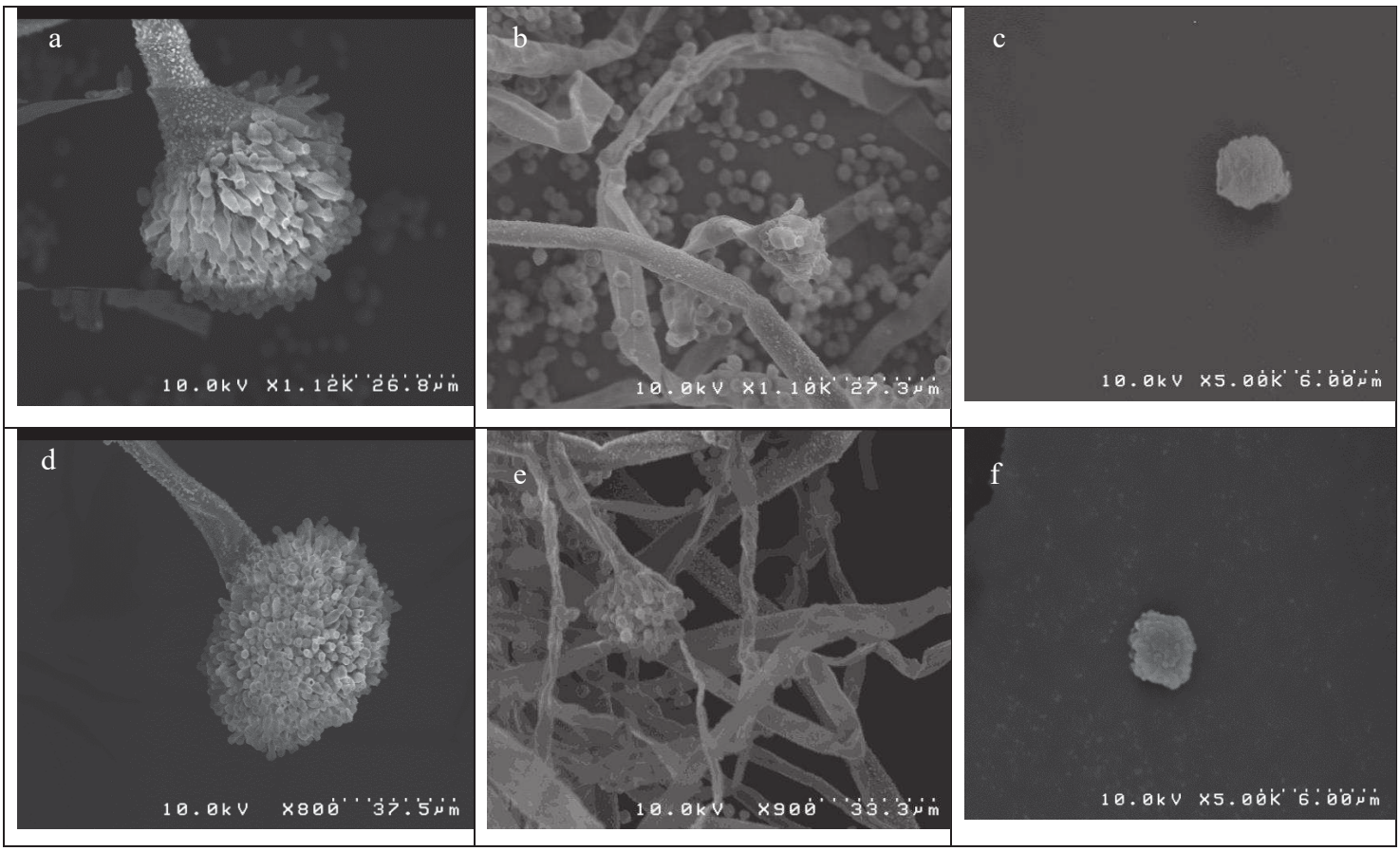


Fig. 3 Scanning electron micrographs (SEM) of *A. flavus* on PDA medium at 25°C for five days: (a) *A. flavus* conidial head. (b) *A. flavus* hyphae. (c) spore of *A. flavus*. (d) *A. flavus* conidial head with 20 g/L of Antoferine®. (e) *A. flavus* hyphae with 20 g/L of Antoferine®. (f) spore of *A. flavus* with 20 g/L of Antoferine®.

Transmission electron microscopy (TEM)

Using TEM, the effects of Antoferine® have been observed at different levels of the ultrastructure of *A. flavus* (see Fig. 4). The key changes included leakage of cytoplasmic contents and the cytoplasm appeared very dense showing wide vacuoles.

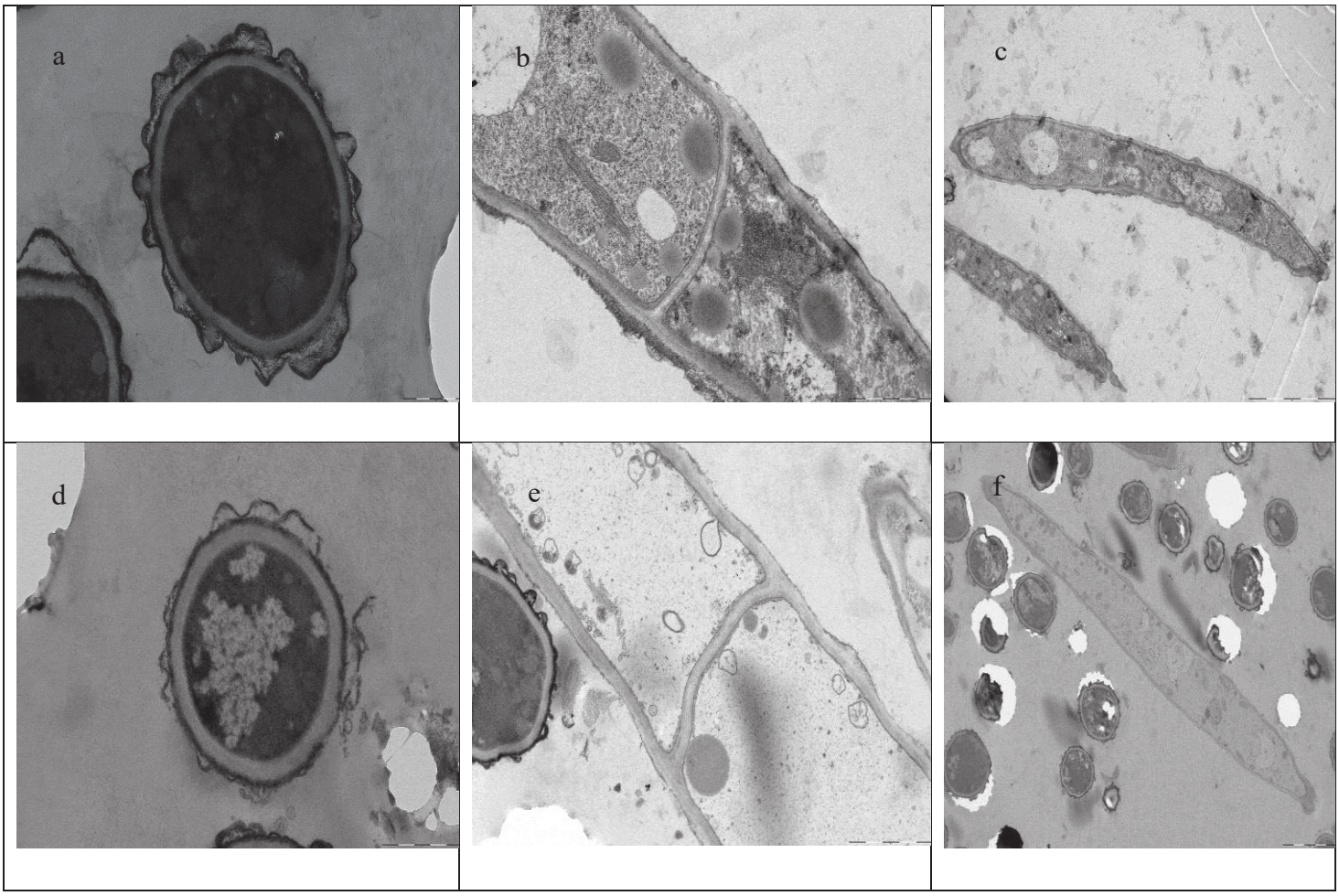
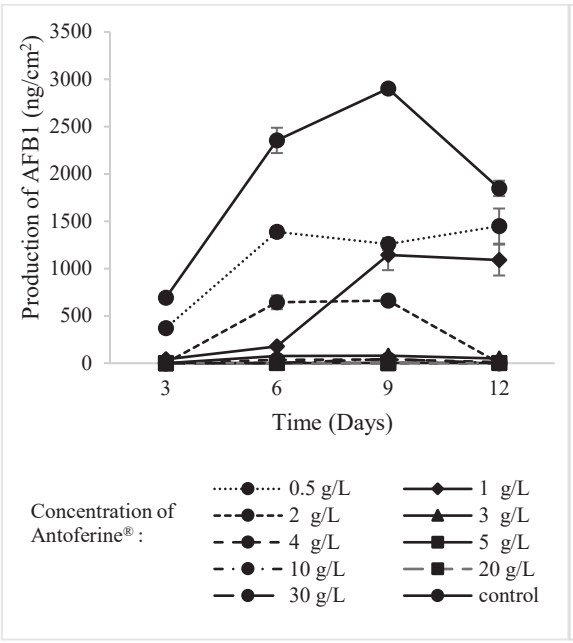


Fig. 4 Transmission electron micrographs (TEM) of *A. flavus* grown on PDA medium at 25°C for five days: (a, b and c) controls. (d, e and f) with 20 g/L of Antoferine®.

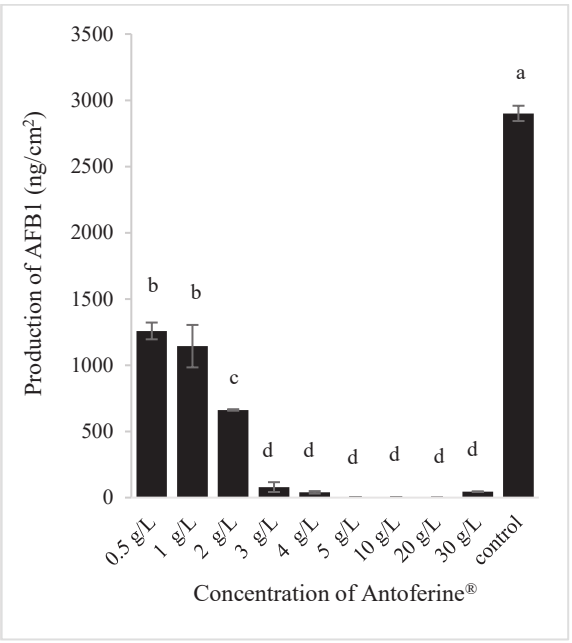
Impact of Antoferine® on mycotoxin production

A kinetic study was carried out with the aim of evaluating the effect of Antoferine® on the production of the mycotoxins AFB1, OTA and FB1 by *A. flavus, A. carbonarius* and *F. verticillioides,* respectively. Mycotoxin production was measured for 12 days, along with the fungal growth, on days 3, 6, 9 and 12. Like it was observed for the fungal growth, Antoferine® affected the production of mycotoxins depending on the nature of the strains and the concentration. The production of AFB1 by *A. flavus* in the cultures implemented with Antoferine®, compared to the control, was concentration-dependent (see Fig. 5 a). At day 9, the results showed a significative ($P<0.01$) decrease of AFB1 production by *A. flavus* grown in PDA medium supplemented with Antoferine®. The effect appeared from the lowest concentration (0.5 g/L) and AFB1 was no longer detectable from the concentration of 5 g/L (see Fig. 5 b). For *A. carbonarius*, the OTA production was diversely impacted by Antoferine® (see Fig. 5 c). No or low inhibition were observed and even an increase of the production was significantly ($P<0.01$) noticed on day 6 for 4 g/L of Antoferine® (see Fig. 5 d). Since the fungal growth of *F. verticillioides* was totally inhibited from the concentration of 10 g/L, the mycotoxin production was only studied until 5 g/L of Antoferine® (see Fig. 5 e) and it was observed a significant inhibition ($P<0.001$) for all the concentrations tested (see Fig. 5 f).

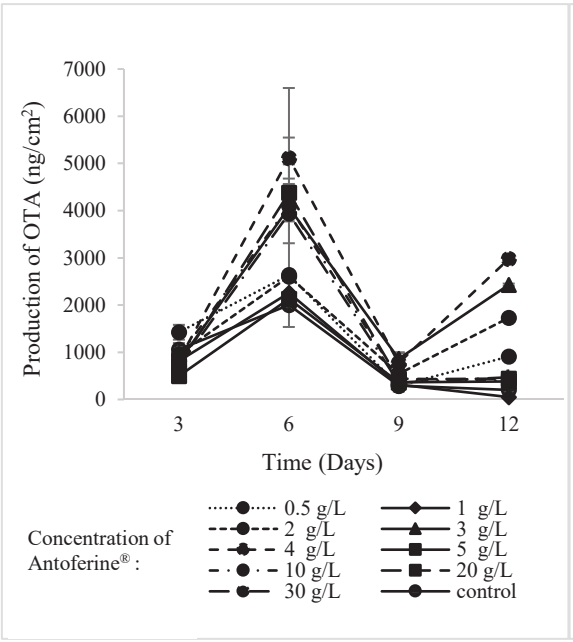
5(a)


Production of AFB1 (ng/cm²)
3500
3000
2500
2000
1500
1000
500
0
3
6
9
12
Time (Days)
Concentration of Antoferine® :
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

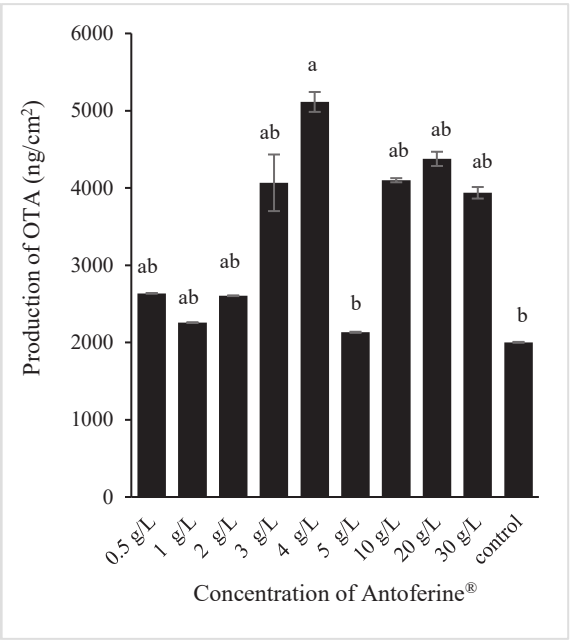
5(b)


Production of AFB1 (ng/cm²)
3500
3000
2500
2000
1500
1000
500
0
b
b
c
d
d
d
d
d
d
a
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control
Concentration of Antoferine®

5(c)


Production of OTA (ng/cm²)
7000
6000
5000
4000
3000
2000
1000
0
3
6
9
12
Time (Days)
Concentration of Antoferine® :
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control

5(d)


Production of OTA (ng/cm²)
6000
5000
4000
3000
2000
1000
0
ab
ab
ab
ab
a
b
ab
ab
ab
b
0.5 g/L
1 g/L
2 g/L
3 g/L
4 g/L
5 g/L
10 g/L
20 g/L
30 g/L
control
Concentration of Antoferine®

5(e) 5(f)

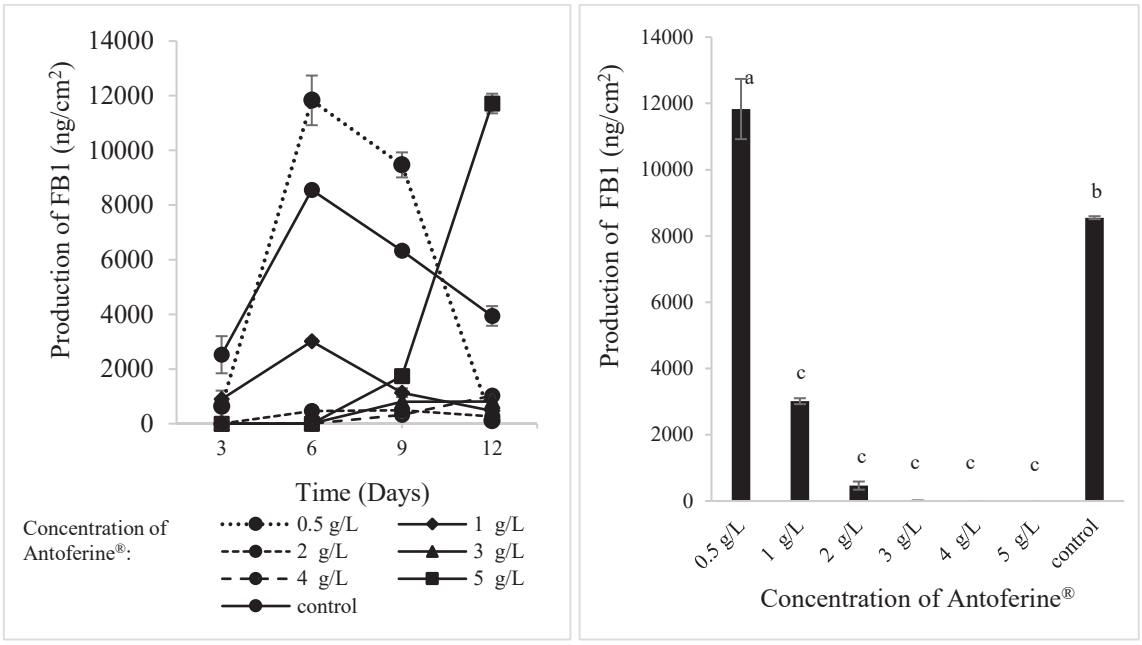
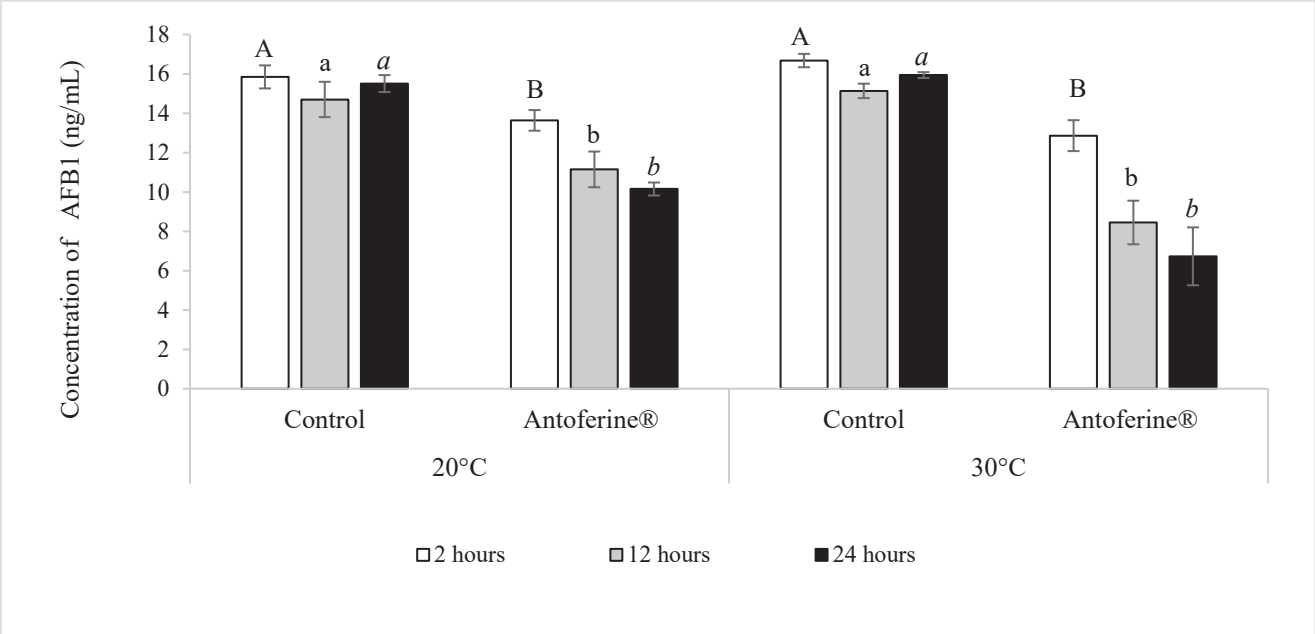


Fig. 5 Effects of different concentrations of Antoferine® on mycotoxin production in PDA medium at 25 °C. (a) Specific production of AFB1 during 12 days. (b) Specific production of AFB1 on day 9. (c) Specific production of OTA during 12 days. (d) Specific production of OTA on day 6. (e) Specific production of FB1 during 12 days. (f) Specific production of FB1 on day 6.

Mycotoxin detoxification

As previous results had shown very little effect of Antoferine® on OTA content, the study of the effect of Antoferine® (0.83 g/L) on detoxification of the mycotoxins AFB1 (16.7 ng/mL) and FB1 (3.3 µg/mL) was carried out at 20 and 30 °C at 3 sampling times (2, 12 and 24 h). It was observed that Antoferine® reduced the concentration of the two mycotoxins, depending on the nature of the mycotoxins, the incubation time and the temperature. The results showed a significative ($P<0.001$) degradation of AFB1 (see Fig. 6 a), mostly after 24 h of incubation at the two temperatures but the degree of reduction was higher at the temperature of 30 °C.
Concerning FB1 (see Fig. 6 b), the effects of Antoferine® were more contrasted since the degradation of FB1 was less significant ($P<0.01$).

6(a)



6(b)

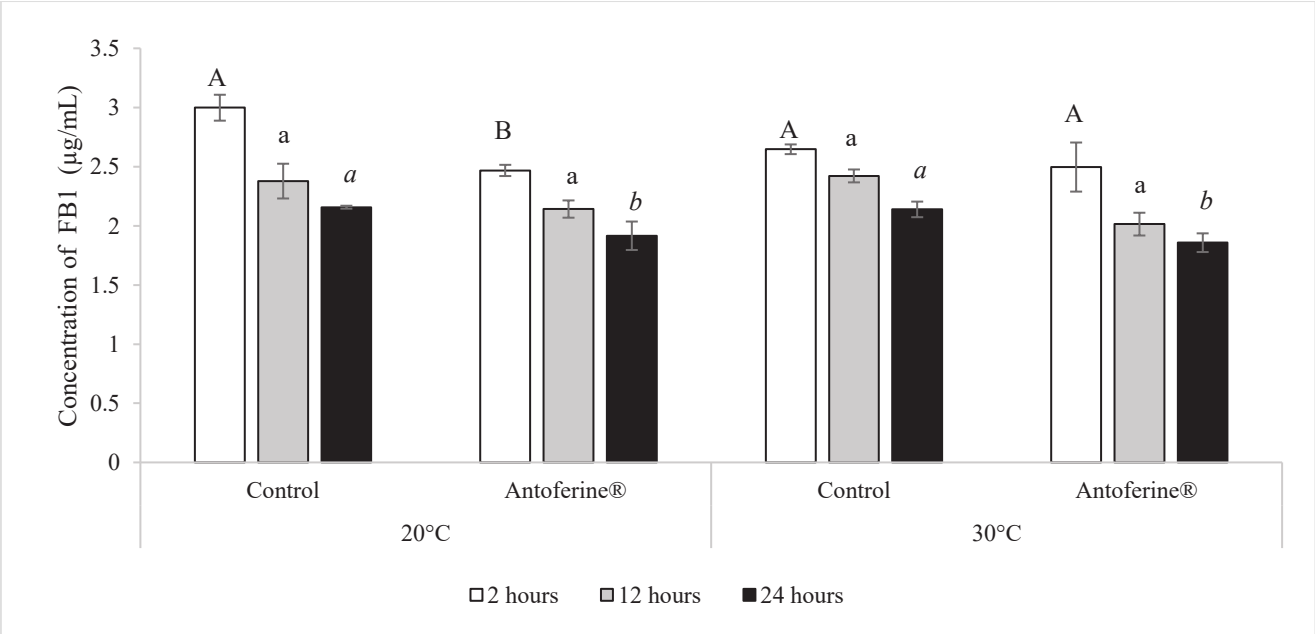


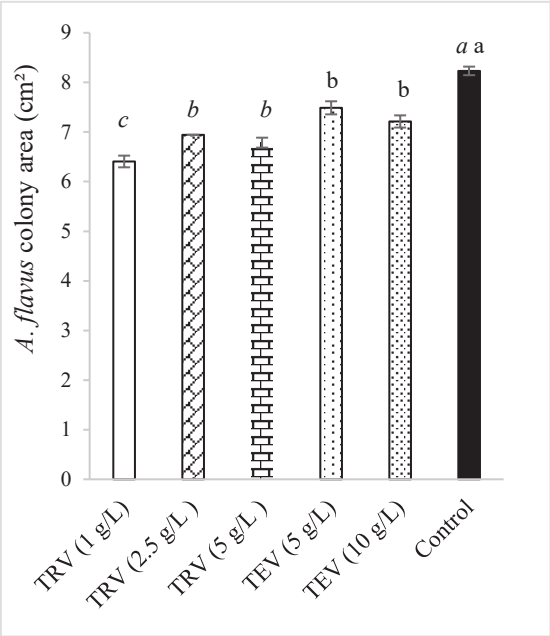
Fig. 6 Detoxification of mycotoxins using Antoferine® 0.83 g/L. (a) detoxification rate of AFB1 16.7 ng/mL. (b) detoxification rate of FB1 3.3 µg/mL.

Antifungal activity and anti-mycotoxigenic effects of the main compounds of Antoferine®

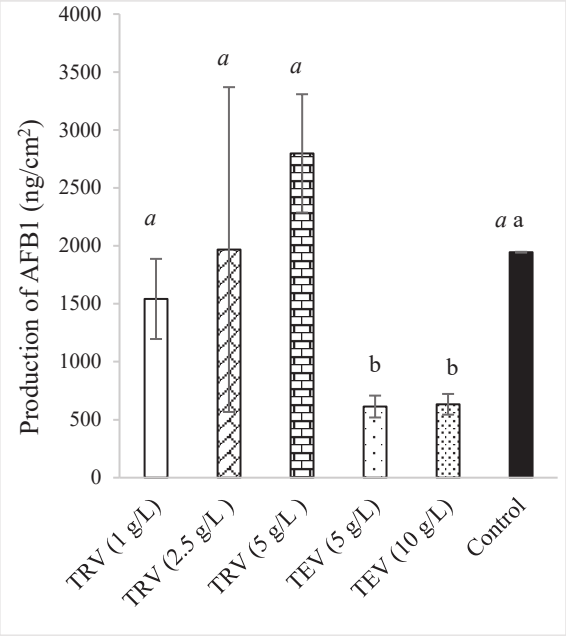
The inhibitory effects of Trans-resveratrol and Trans-Ɛ-viniferin on the growth of *A. flavus, A. carbonarius* and *F. verticillioides* and the production of their mycotoxins were determined in PDA agar at 25 °C for 4 days. The results showed that the two compounds affected the growth of fungi and their production of mycotoxins, depending on the strains and the concentration of the compounds.

A significative ($P<0.001$) inhibition of the growth of *A. flavus* (see Fig. 7 a), using Trans-resveratrol at the concentration of 1 g/L was observed, while AFB1 production (see Fig. 7 b) was not affected. Trans-Ɛ-viniferin impacted growth and AFB1 content at the 2 concentrations tested. Only Trans-resveratrol reduced the growth of *A. carbonarius,* especially at the 1 g/L concentration (see Fig. 7 c). OTA production was significantly ($P<0.001$) impacted by Trans-resveratrol at the concentrations of 2 and 5 g/L but Trans-Ɛ-viniferin stimulated the production of OTA at both concentrations tested (see Fig. 7 d). The growth of *F. verticillioides* was reduced in the presence of all three concentrations of Trans-resveratrol while Trans-Ɛ-viniferin was only efficient at 5 g/L (see Fig. 7 e). The production of FB1was particularly impacted by Trans-resveratrol at the concentration of 1 g/L and Trans-Ɛ-viniferin at the concentration of 5 g/L (see Fig. 7 f).

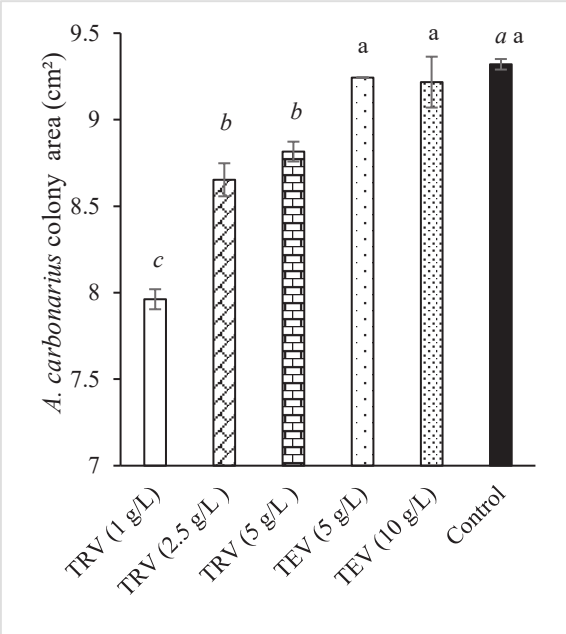
7(a)


A. flavus colony area (cm²)
0
1
2
3
4
5
6
7
8
9
c
b
b
b
b
a a
TRV (1 g/L)
TRV (2.5 g/L)
TRV (5 g/L)
TEV (5 g/L)
TEV (10 g/L)
Control

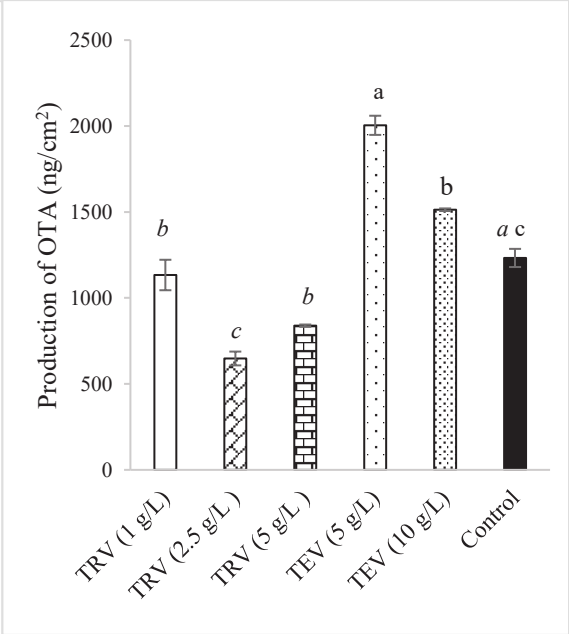
7(b)


Production of AFB1 (ng/cm²)
0
500
1000
1500
2000
2500
3000
3500
4000
a
a
a
b
b
a a
TRV (1 g/L)
TRV (2.5 g/L)
TRV (5 g/L)
TEV (5 g/L)
TEV (10 g/L)
Control

7(c)


A. carbonarius colony area (cm²)
7
7.5
8
8.5
9
9.5
c
b
b
a
a
a a
TRV (1 g/L)
TRV (2.5 g/L)
TRV (5 g/L)
TEV (5 g/L)
TEV (10 g/L)
Control

7(d)


Production of OTA (ng/cm²)
0
500
1000
1500
2000
2500
b
c
b
a
b
a c
TRV (1 g/L)
TRV (2.5 g/L)
TRV (5 g/L)
TEV (5 g/L)
TEV (10 g/L)
Control

7(e) 7(f)

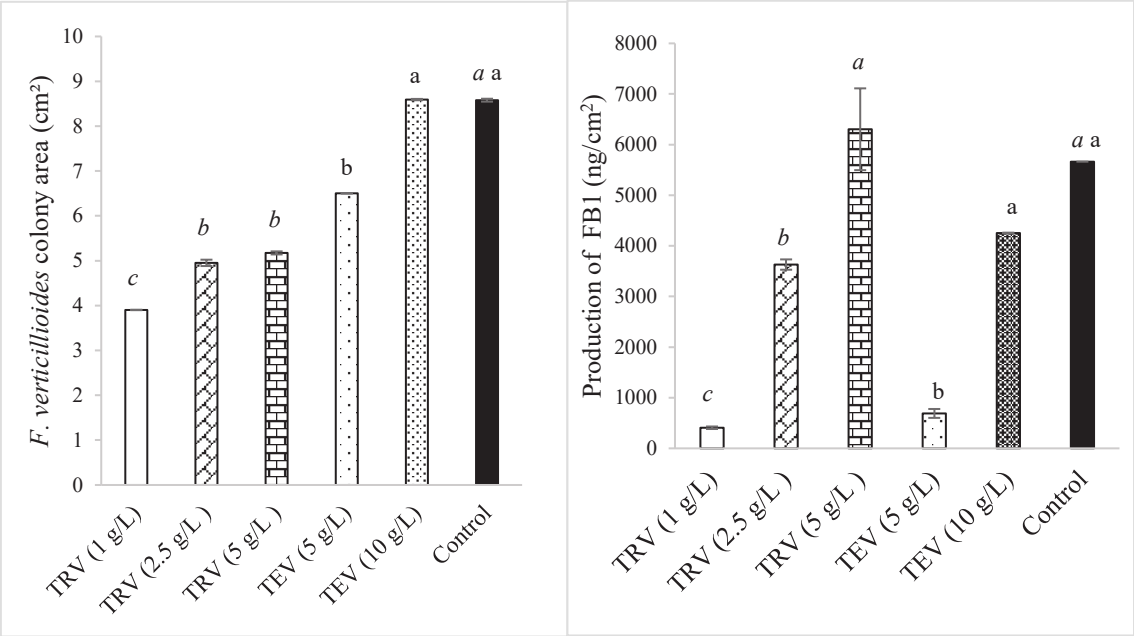
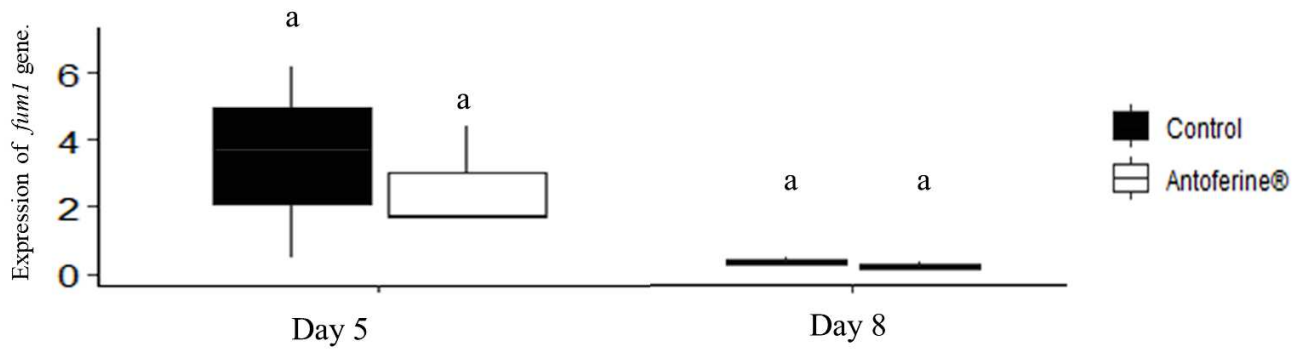


Fig. 7 Effect of different concentrations of Trans-resveratrol (TRV) and Trans-Ɛ-viniferin (TEV) on colony surface (cm²) and mycotoxin production in PDA medium at 25 °C for 4 days. (a) on colony surface of *A. flavus*. (b) on AFB1 produced by *A. flavus.* (c) on colony surface of *A. carbonarius.* (d) on OTA produced by *A. carbonarius*. (e) on colony surface of *F. verticillioides*. (f) on FB1 produced by *F. verticillioides.*

RT-qPCR analysis of the expression of genes involved in aflatoxin B1 and fumonisin B1 biosynthesis

The ability of Antoferine® (1 g/L) to modulate the expression levels of genes involved in the AFB1 and FB1 biosynthetic pathways were examined using RT-qPCR. The examined genes were *aflD* and *aflP* for *A. flavus* and *fum1* and *fum8* for *F. verticillioides*. The genes *TBAf* and *TBFv* (β-tubuline) were used to normalize the gene expression. After 5 or 8 days of growth in the presence of Antoferine®, there has been no change in gene expression of *fum1* and *fum8* (see Fig. 8). Concerning *A. flavus*, the genes were not affected by the presence of Antoferine® at day 5 but *aflP* was down regulated and *aflD* was upregulated in comparison to the control at day 8 (see Fig. 9).

8(a)



8(b)

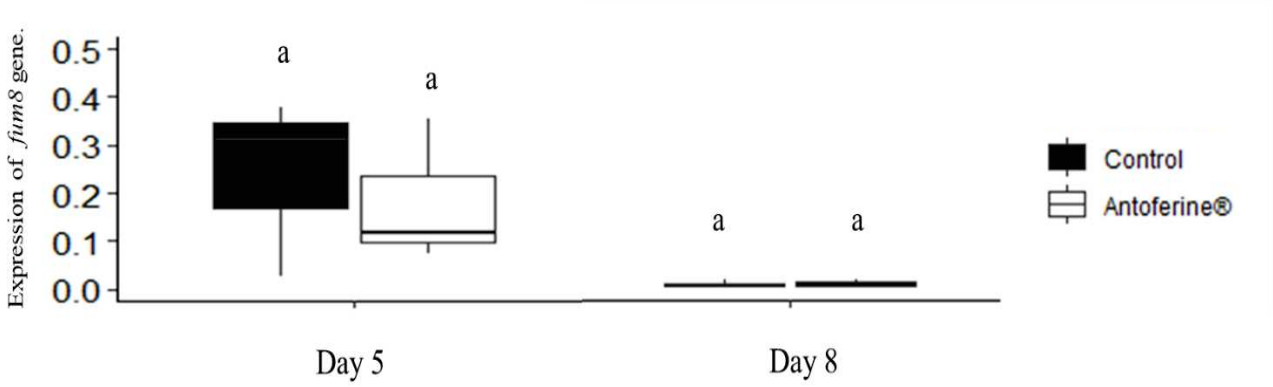
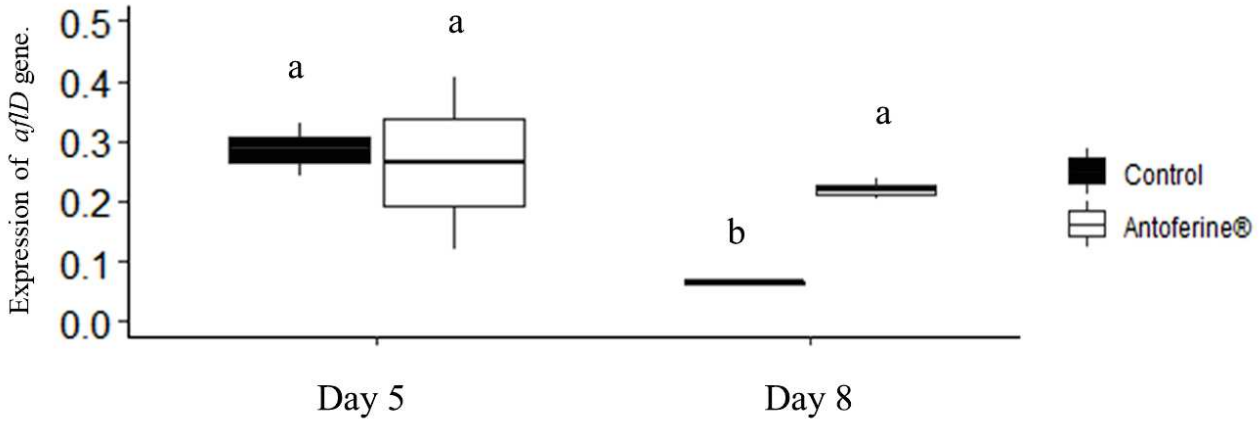


Fig. 8 Expression of genes involved in fumonisin B1 biosynthesis in *F. verticillioides* grown with 1 g/L of Antoferine® and incubated at 25 °C in PDA medium for 5 or 8 days. (a) Expression of *fum1* gene, (b) Expression of *fum8* gene. Different letters above the same column indicate significant differences from the control group ($p < 0.05$).

9(a)



9(b)

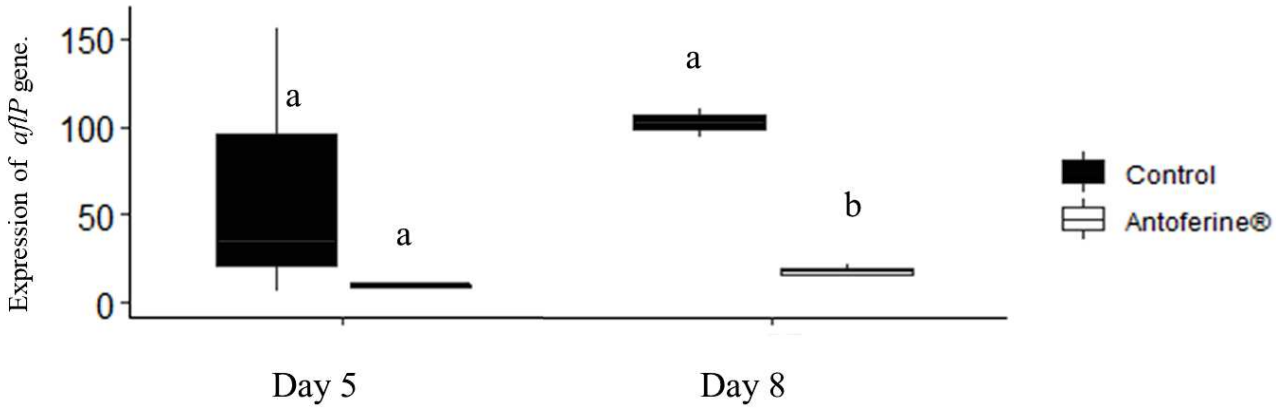


Fig. 9 Expression of genes (*aflD* and *aflP*) involved in Aflatoxin B1 biosynthesis in *A. flavus* grown with 1 g/L of Antoferine® and incubated at 25 °C in PDA medium for 5 or 8 days. (a) Expression of *aflD* gene, (b) Expression of *aflP* gene. Different letters above the same column indicate significant differences from the control group ($p < 0.05$).

Discussion

On the basis that the use of plant extract and/or their bioactive components is a promising approach, (Rachelle El Khoury et al., 2016) the aim of this study was to evaluated the capacity of Antoferine® to impact the growth and mycotoxinogenesis of *A. flavus*, *A. carbonarius* and *F. verticillioides*. Overall, contrasting effects were observed according to the different fungal strains studied.

Concerning the growth and sporulation, the colony surface of *A. flavus* was significantly impacted by Antoferine® from the concentration of 2 g/L. However, the impact on sporulation is less clear since the concentration of 10 g/L increased the production of spores while other concentrations decreased more or less sporulation. Elsewhere, antifungal and anti-mycotoxigenic properties of Antoferine® are likely to be effective for containing phenolic compounds. Phenolic compounds are characterized by their lipophobicity structure that allows them to penetrate the cell membrane of fungi (Loi et al., 2020). This leads to disrupting cell membrane permeability and functions, to inhibiting cytoplasmic and mitochondrial enzymes, to inhibiting enzymes involved in cell wall components synthesis and to altering the cell compartment, the osmotic and redox balance (Jayashree & Subramanyam, 2000). These events induce cytotoxicity in fungi and deformations of structures which were observed in A. flavus using the electron microscope after treatment with Antoferine®. Similarly, Wang et al. (2019) found morphological changes in *Alternaria alternata* after exposure to citral essential oil. *F. verticillioides* colony surface was significantly impacted by Antoferine®. Interestingly, the growth was completely hampered from the concentration of 10 g/L. A significant decrease of spore production was noticed using the concentration of 5 g/L. These results were similar to those obtained by Xing et al. (2014) who showed that the utilization of natural cinnamaldehyde at 20 mL/L could inhibit the growth of *F. verticillioides*. Unlike the other 2 strains, *A. carbonarius* growth, as well as its spore production, were very few impacted by Antoferine®.

Antoferine® has been able to inhibit significantly AFB1 production from 0.5 g/L. These results were similar to those obtained by Caceres et al. (2017) who found that the use of piperine, the main component of black and long peppers (*Piper nigrum* L. and *Piper longum* L.), induced an AFB1 inhibition of 95% at the concentration of 0.04 mM, with impacting moderately the fungal mycelial growth of *A. flavus*. Production of FB1 was also strongly inhibited. This result was similar to those obtained by Dambolena et al. (2011) that showed that the utilization of ten phenolic compounds inhibited the production of FB1 by *F. verticillioides*, with thymol,

carvacrol, isoeugenol and eugenol being the most active antifumonisin compounds. Contrary to the results obtained with AFB1 and FB1, Antoferine® has significantly stimulated OTA production, and these results were not consistent with those obtained by Crespo-Sempere et al. (2016) who found that polyphenol extract of grapes was able to reduce significantly the content of OTA (22%) at the highest concentration assayed (35 ppm).

On the basis that plant extracts could also act by inducing mycotoxins detoxification,(Wu et al., 2017) the experiments carried out have shown that the concentration of 0.83 g/L Antoferine® was capable to significantly reduce the content of AFB1. Similarly, Ponzilacqua et al. (2019) noticed aqueous extracts of sweet passion fruit (*Passiflora alata*), araçá (*Psidium cattleianum*), rosemary (*Rosmarinus officinalis*) and oregano (Origanum vulgare) for their ability to degrade AFB1 in vitro. The phenolic compounds can transform AFB1 to a fewer toxic compound by double-bond eradication from a furan ring plus the readjustment for the lactone-ring structure. Natural compounds such as polyphenols like stilbenes and flavonoids are able to inhibit cytochrome P450 enzymes involved in the bioactivation mechanisms of xenobiotics like mycotoxins. In some cases, these enzymes can generate toxic metabolites like mutagenic aflatoxin-8,9-epoxide from aflatoxin B1. On the other hand, these compounds can induce enzymes such as glutathione S-transferases, UDP-glucuronosyl-transferases or sulfotransferases that are associated with the detoxification of reactive xenobiotic metabolites and their excretion(Galvano et al., 2001; Wu et al., 2017). Antoferine® was able also to slightly reduce the concentration of FB1 at 20 °C and 30 °C. These results were similar to those obtained by Xing et al. (2014) which reported that cinnamon oil degraded FB1 at different incubation times, temperatures and concentrations.

What also characterize phenolic compounds is their antioxidant potential. Numerous studies have shown relations between antifungal and antimycotoxigenic potential of natural extracts and contents in total phenolics and antioxidant potential. Several physiological mechanisms in fungal cells are regulated by oxidative changes, including the secretion of components of secondary metabolism. On the one hand, oxidative stress is capable of promoting mycotoxin biosynthesis (Jayashree & Subramanyam, 2000). Conversely, the presence of antioxidant phenolic compounds alters the growth conditions of fungi, which can affect fungal metabolism and limit mycotoxin formation. Indeed, phenolic compounds are known to impact the expression of genes in the mycotoxin biosynthetic pathway (Badr et al., 2020; Riihinen et al., 2008).

As a result of the experiments carried out with the main components of Antoferine®, it was observed that the antifungal and anti-mycotoxin activities of Antoferine® could not be ascribed to the action of a single active molecule, but to the result of synergistic interactions between several compounds. Indeed, some weak inhibitory effects were observed on the growth of 3 fungal strains using the compounds separately. Neither of the two main Antoferine® compounds achieved the same levels of inhibition as observed with Antoferine® itself.

For *A. flavus*, *aflD* was upregulated while *aflP* was downregulated in comparison to the control at day 8. The latter result was in line with many studies showings that the use of plant extracts could downregulate mycotoxin biosynthesis genes. For example, El Khoury et al. (2017) showed that the use of an aqueous extract of the medicinal plant *Micromeria graeca*, known as hyssop, resulted in a repression of all AFB1 biosynthesis genes. Another study by Lv et al. (2018) showed that eugenol strongly decreased most AFB1 biosynthetic genes in *A. flavus* and among these genes were *aflD* and *aflP*. For *F. verticillioides*, no change in the expression of *fum1* or *fum8* was noted. Expression of *fum1* correlates directly with fumonisin biosynthesis, whereas *fum8* is instead involved in the chemotype change of fumonisin during synthesis. This gene encodes a ″α-oxoamine synthase″ that catalyzes the condensation of the synthesizing polyketide with alanine or glycine to produce FB1 or FB2, respectively, thus mediating between fumonisin B1 and B2 (Proctor et al., 2013). However, in order to obtain
more accurate results, it would be useful to study a larger number of genes on more different days.

Conclusion

Antoferine® seems to be a promising solution for the biocontrol certain toxigenic fungi. It could represent an eco-friendly alternative to chemical fungicides to control the mycotoxic risk. However, these interesting effects must be confirmed in field trials. An extended study to other mycotoxigenic strains and the deciphering of the mechanisms of action are now to be considered.

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Declarations

Funding Not applicable

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Availability of data and material Not applicable

Code availability Not applicable

Authors' contributions All authors have read and agree to the published version of the manuscript. Asma Chelaghema, Angélique Fontana and Caroline Strub designed the study. Noël Durand performed mycotoxin analysis via HPLC. Christophe Jourdan participated in manipulation of qPCR. Asma Chelaghema carried out the entire range of manipulations and experimental approaches and analyzed the data. Asma Chelaghema, Caroline Strub, and Angélique Fontana interpreted the data. Asma Chelaghema wrote the original draft. Angélique Fontana, Caroline Strub and Sabine Schorr-Galindo carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

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Conclusion

L'Antoferine® a montré une activité antifongique contre *A. flavus*, *F. verticillioides*. Les résultats ont montré également sa capacité à inhiber la production des mycotoxines (aflatoxine B1 (AFB1) et fumonisine B1 (FB1) produites par *A. flavus* et *F. verticillioides*, respectivement). D'autre part, il est intéressant de noter une activité différente de l'Antoferine® vis-à-vis d'*A. carbonarius*. En effet, parallèlement à un faible effet sur la croissance, l'Antoferine® a stimulé la production d'ochratoxine A (OTA).

Les principaux composants bioactifs (Trans-resveratrol et Trans-Ɛ-viniférine) ont donné des résultats différents selon les souches et les concentrations utilisées. Il est apparu que les activités antifongiques et antimycotoxiques de l'Antoferine® résultent de l'action conjointe de ces deux composés et des autres molécules qui composent l'extrait végétal.

L'Antoferine® pourrait être une solution prometteuse pour limiter le risque mycotoxique mais il est nécessaire d'élargir l'étude à d'autres souches fongiques mycotoxinogènes et de vérifier au champ les effets observés *in vitro*. Dans un objectif d'homologation du produit, l'étude de ses mécanismes d'action devra également être approfondie.

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2.2 Activité antifongique et anti-mycotoxinogène des huiles essentielles de *Cymbopogon nardus, Cymbopogon schoenanthus* et *Eucalyptus camaldulensis*

Introduction

Des efforts sont déployés dans le monde entier pour remplacer les pesticides chimiques synthétiques par des alternatives, qui sont plus sûres pour l'environnement, l'utilisateur et le consommateur. La lutte naturelle contre les ravageurs et les maladies, directement ou indirectement à l'aide de produits végétaux/botaniques naturels, y compris les huiles essentielles (HE), est très prometteuse (Batish et al., 2008).

Les huiles essentielles sont des produits aromatiques et volatils du métabolisme secondaire des plantes, et peuvent être trouvées notamment dans les feuilles et les tiges (Chou et al., 2018).

Leur activité antifongique est liée à la structure chimique de leurs composants actifs. En effet les HE sont des mélanges de molécules caractérisées par leur faible solubilité dans l'eau et leur forte hydrophobie. Cette hydrophobie facilite leur incorporation dans les membranes plasmiques et celles des organites intracellulaires (notamment les mitochondries). Ces composés peuvent alors agir en altérant la composition de la membrane lipidique, en diminuant des taux d'ergostérols par exemple (Chelaghema et al., 2021). Leur activité vis-à-vis des organismes vivants est également dose-dépendante, c'est-à-dire qu'elle est liée à la concentration à laquelle elles sont employées.

Les HEs sont constituées par une grande variété de composés actifs, qui leur confèrent un large spectre de modes d'action en leur donnant la capacité de cibler plusieurs fonctions au niveau de la cellule fongique.

C'est dans ce contexte que l'étude du potentiel antifongique et/ou antimycotoxique de 3 huiles essentielles a été réalisée sur 3 souches fongiques d'intérêt. Ainsi, cette étude est une contribution à la caractérisation chimique des huiles essentielles de *Cymbopogon schoenanthus, Cymbopogon nardus* et *Eucalyptus camaldulensis* et de leurs effets sur la croissance et la mycotoxinogenèse d'*Aspergillus flavus, Aspergillus carbonarius* et *Fusarium verticillioides*, respectivement producteurs d'aflatoxine B1 (AFB1), d'ochratoxine A (OTA) et de fumonisine B1 (FB1). De plus, leurs effets sur l'expression de certains gènes responsables de la biosynthèse de l'aflatoxine B1 et de la fumonisine B1 a été examinée.

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Antifungal and antimycotoxic activities of 3 essential oils against 3 mycotoxinogenic fungi

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Abstract

Fungal toxins can have various adverse health effects, including carcinogenic, teratogenic or hepatotoxic impacts. In addition, fungal alteration has also a negative impact on agricultural plant production. The use of chemical fungicides to control mycotoxin contamination is increasingly controversial and regulated. More environmentally friendly methods are therefore being explored. Essential oils, as compounds extracted from plants, are liquids whose specific aromatic compounds give each essential oil its own unique characteristics. Due to their rich chemical composition, essential oils (EOs) have many interesting properties, including antifungal activities. The objective of the present study was to analyze volatile chemical composition of EOs (*Cymbopogon schoenanthus, Cymbopogon nardus* and *Eucalyptus camaldulensis*) by GC/MS and to investigate their effects on the growth, sporulation and mycotoxin production of *Aspergillus flavus, Aspergillus carbonarius* and *Fusarium verticillioides* (aflatoxin B1, ochratoxin A and fumonisin B1, respectively). In addition, EOs influence on aflatoxin B1 (AFB1) and fumonisin B1 (FB1) biosynthesis pathways was explored using real time qRT-PCR.

The results obtained in vitro, by direct contact with the EOs and by diffusion of their volatile compounds, showed that the essential oils had inhibitory effects on the growth and the production of mycotoxins of the 3 fungal strains and modified the expression of some toxin synthesis genes.

We conclude that the recorded effects were dependent on the combined effects of the Eos type, the fungal strains and the doses studied.

Keywords Essential oils, biocontrol, mycotoxins, fungi.

Introduction

Essential oils extracted from plants are widely used by humans in various fields, including pharmaceutical, cosmetic, food and nutritional (Ganjewala, 2009). They contain a wide variety of natural substances among which secondary metabolites of interest for these various applications (Chaudhari et al., 2019).

Agricultural losses due to phytopathogenic agents are recorded all over the world and represent a serious socio-economic burden. A significant number of diseases affecting plants are of fungal origin (Perczak et al., 2020). Among these, one of the most damaging diseases is the "Fusarium wilt of cereals", which is usually caused by *Fusarium* species on small grains such as wheat, barley, oats and maize. It causes necrosis on different vegetative parts of the affected plant, generating significant quality and yield losses potentially leading to the waste of the entire crop (Nguyen et al., 2017). *Fusarium* spp are also able to produce mycotoxins, secondary metabolites that can generate serious hazards for human and animal health (Dadzie et al., 2019; Lee & Ryu, 2017). These mycotoxins produced by *Fusarium* sp. are called fusario-toxins (trichothecenes, zearalenone, and fumonisins) (Bertero et al., 2018).

Fungal strains of the genus *Aspergillus* are also responsible for the accumulation of mycotoxins in many food plant productions such as cereals and cereal by-products, grapes and grape by-products, spices, coffee, cocoa or nuts and peanuts (El Khoury et al., 2016; El Khoury et al., 2017; Pickova et al. 2020). The most important mycotoxins produced by *Aspergillus* strains are aflatoxin B1 and ochratoxin A. They are considered as carcinogenic or potentially carcinogenic to all animal species (Haque et al., 2020; IARC, 2002; Ostry et al., 2017)

The use of chemical fungicides remains the most widespread means for controlling phytopathogenic and/or mycotoxinogenic fungal strains because of their ease of application and their persistence (Dikhoba et al., 2019). However, current concerns about the impact of pesticides on human or animal health and on the environment are leading to a ban on the use of some pesticides as well as to the consideration of more responsible solutions (Moraes Bazioli et al., 2019).

Therefore this situation paved the way for research and future adoption of alternative control methods, such as those based on the use of natural plant-based compounds like essential oils (D'agostino et al., 2019). The use of essential oils in plant protection is still in its beginning but these products have promising fungicidal potential as a viable alternative to synthetic fungicides. Essential oil-based biofungicides are being marketed for organic farmers. These are

SporanTM (*Rosmarinus officin nalis*), PromaxTM (*Thymus vulgaris*), TrilogyTM (*Acalypha indica*) and E-RaseTM (*Simmondsia californica*) (Ncama et al., 2019).

In this perspective we carried out the evaluation of the antifungal and/or antimycotoxic potential of 3 essential oils on 3 fungal strains of interest. The present work aimed at the characterization of the chemical profile of *Cymbopogon schoenanthus*, *Cymbopogon nardus* and *Eucalyptus camaldulensis* essential oils and their effects on the growth and toxinogenesis of *Aspergillus flavus*, *Aspergillus carbonarius* and *Fusarium verticillioides*, respectively producers of aflatoxin B1, ochratoxin A and fumonisin B1. In addition, the effects of these essential oils on the expression of certain genes responsible for the biosynthesis of aflatoxin B1 and fumonisin B1 was explored.

Materials and methods

Preparation of spore suspensions

All fungi were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) under paraffin oil at 4 °C and actively grown on PDA at 25°C for 7 days for spore production. Spore suspensions were prepared by adding 10 mL of a sterile distilled water to the plate then by scrapping the surface of the cultures with a sterile spreader. The spore suspensions were collected and theirs spores concentration were determined using Thoma cell counting chamber. Spore concentrations were then adjusted to 10^6 spores/mL.

Culture medium and microorganism

Toxigenic filamentous fungal strain *A. flavus* E73 (UMR Toxalim, Toulouse, France), *A. carbonarius* (property of UMR QualiSud, Montpellier, France) *and F. verticillioides* BRFM 2251 (CIRM, University of Aix-Marseille, Marseille, France) were chosen because of their strong ability to produce their respective mycotoxins (Ahmed et al., 2015b; Campos-Avelar et al., 2020; Pellan et al., 2020)

All fungi were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) under paraffin oil at 4 °C. Inoculum was prepared by surface washing of the actively grown molds sub-cultured on PDA agar medium for 7 days at 25°C. After the incubation period, a spore suspension was prepared by adding 10 mL of a sterile physiological water to the surface of the culture, followed by a gentle scrapping. The evaluation of the spores concentration was conducted using Thoma cell counting chamber that helped to adjust the concentration to 10^6 spores/mL.

Essential oils (EOs)

The 3 essential oils (EOs), *Cymbopogon schoenanthus*, *Cymbopogon nardus* and *Eucalyptus camaldulensis,* were purchased from a local market in Burkina Faso and were stored at 4°C before the experiments. These EOs were chosen because of their antifungal effects described in the literature (Abo Elgat et al., 2020; Dwivedy et al., 2016; Gakuubi et al., 2017). They were also selected because of their availability on local markets in areas particularly affected by mycotoxin contamination.

Gas chromatographic (GC) and gas chromatographic/mass spectrometry (GC/MS) analysis

Briefly, 1.5 g of homogenized sample was placed and sealed in a 10 mL head-space vial and extracted by headspace - solid phase micro extraction (HS-SPME) (Abbasi et al., 2020; Devi et al., 2021; Scurria et al., 2020). A tandem gas chromatograph 6890/MSD 5973 N (Agilent Technologies, USA) and a Gerstel autosampler MPS2 were used. The entrapment was realized under orbital shaking for 45 min with DVB (divinyl benzene) – CAR (carboxen) – PDMS (polydimethylsiloxane) fiber 65µm (Supelco, Bellefonte, USA) at 50 °C after 10 min of incubation. A polar capillary DB-WAX polar column (60 m, 0.25 mm, 0.25 µm phase film thickness) from J&W Scientific (Folsom, CA, USA) was used with a carrier gas hydrogen set at a flow rate of 1.5 mL/min. Fiber was desorbed at 250 °C and compounds were separated with the following temperature program: the initial oven temperature was 40°C (5 min isotherm), subsequently increased by 2 °C/min to 140 °C, and then by 10 °C/min to 250 °C, and maintained for 10 min. Mass spectra were recorded in EI+ mode at 70 eV within a range of 40 to 350 Da with a solvent delay time of 2 min and scan speed of 4.52/s. Analyzer and source temperatures were 150 °C and 250 °C, respectively. Data were analyzed with Mass hunter version B. 06.00 (Agilent Technologies, USA). NIST Database 2011 (National Institute of Standard Technology) were chosen to identify peaks by comparison with their mass spectra. Results were expressed in relative percent.

Inhibition of fungal growth

In order to study the antifungal activity of EOs, against fungal strains. The effects of the 3 EOs extracts was evaluated using two methods:

Agar diffusion method

The antifungal activities of essential oils were determined by measuring the fungal colonies area exposed to EOs. EOs were mixed with PDA medium up to final concentrations (0.pr, 0.5 and 1 µL/mL). Essential oils-free PDA medium was used as control. All the treatments and control were inoculated with 5 µL of a spore suspension adjusted to 10^6 spore/mL of each fungal

strain in the center of the plate and incubated in the dark at 25°C, for 12 days. Every 3 days, Petri dish were photographed, and the colony area of each fungus were measured in cm^2 using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018).

Disc volatilization method

PDA medium was firstly inoculated with fungal suspension in the center of the plate (5 µL of spore suspension adjusted to 10^6 spores/mL). Essential oils were then pipetted on the paper disc (diameter 6 mm) and placed on the inside surface of the upper lid of Petri dishes. The lids were immediately closed and sealed with parafilm to prevent leakage of the EOs vapour. Sterile water was used as control. Petri dishes were then incubated in the dark at 25°C for 12 days. Every 3 days, Petri dish were photographed and the colony area of each fungus were measured in cm^2 using ImageJ software. All the treatment and control were inoculated in triplicate.

Mycotoxin extraction and quantification of fumonisin B1, ochratoxin A and aflatoxin B1

Every 3 days cultures and for 12 days, the extraction from the PDA medium were done by removing the half of a Petri dish agar from each culture condition as well as the control, placed in pot and weighed. Then the culture media were mixed with 25 mL and 50 mL of a methanol/formic acid mixture (25:1, v/v) (VWR, Fontenay-Sous-Bois, France) for *A. carbonarius* and *A. flavus.* respectively, and 50 mL of water/acetic acid (99.5:0.5, v/v/v) for *F. verticillioides* cultures. All samples were homogenized by mechanical agitation using an orbital shaker at 250 rpm for 20 min. Extraction solvent from *F. verticillioides* samples were filtered directly with a CA filter 0.45 µm (Carl Roth GmbH, Karlsruhe, Germany). A total of 500 µL of the mixture from Aspergilli samples were evaporate until dryness at 60 °C in SpeedVac vacuum concentrator (Eppendorf® AG, Hamburg, Germany). Residues were taken up in 2 mL of solvent corresponding to the mobile phase used for ochratoxin HPLC analysis, and for aflatoxin HPLC analysis then placed in an ultrasonic bath for 20 minutes and filtered through a 0.45 µm PTFE filter (Dominique Dutscher S.a, Grabels, France).

Sample analysis was performed with Ultra High-Performance Liquid Chromatography (Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan) for the quantification of Fumonisin B1. The operating conditions were: injection volume of 50

µL; HPLC column C_{18} Kinetex XB 50 × 2 mm, 2.6 µm particles size (Phenomenex, California, USA); column temperature at 50°C; and a constant flow rate of 0.4 mL/min. The mobile phase consisted of 0.5% acetic acid in ultra-pure water (A) and 0.5% acetic acid in isopropanol (B) (HPLC MS grade, Sigma, St Louis, MO, USA).

The mobile phase gradient program was started at 90% A (0.01 min), 45% A at 1.5 min, 15% A at 3.5 min, 20% A at 4 min, 98% A at 4.01 min and finally 98% A at 11 min.

The mass spectrometer was operated in electrospray positive (ESI^+) and negative (ESI^-) ionization mode, and two multiple reaction monitoring (MRM). The transitions for each analyte were monitored for quantification (Q) and qualification (q) (Table 1). All data were analyzed using LabSolution Software (v5.91/2017, Shimadzu, Tokyo, Japan, 2017). Limits of detection and quantification (LOD/LOQ in ng/mL, respectively) FB1 were (0.03/0.1) (Pellan et al., 2020).

Aflatoxin concentration was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan), after derivatization post column with electrochemical system (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). The operating conditions were set as follows: injection volume of 100 µL; HPLC column C_{18}, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40 °C; isocratic flow rate of 0.8 mL/min; mobile phase: water-methanol (55:45; v/v) with 119 mg/L of potassium bromide and 350 µL/L of nitric acid; excitation wavelength of 365 nm and emission wavelength of 435 nm. Detection and quantification limits for Aflatoxin B1 were established at 0.015 and 0.05 ng/mL respectively (Campos-Avelar et al., 2020). Mycotoxin levels were expressed in ng/cm, specific mycotoxin production.

OTA was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan). The operating conditions were set as follows: injection volume of 100 µL; HPLC column C_{18}, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 35 °C; isocratic flow rate of 1 mL/min; mobile phase: methanol/water/acetic acid, 69:30:1; excitation wavelength of 333 nm and emission wavelength of 460 nm. Detection and quantification limits were established at 0.015 and 0.05 ng/mL respectively.

Table 1 MS/MS parameters for isotope labelled, internal standard (IS) and FB1.
(Q) Quantification; (q) qualification.

	Polarity	MRM Q	EC MRM Q	MRM q	EC MRM q	R^2 calibration curve
U-($^{13}C_{34}$)- FB_1	+	756.3 > 356.5	-47			
FB_1	+	722.35 > 334.5	-43	722.35 > 352.0	-44	0.9982

Effect of essential oils on fungal sporulation

To study the effects of essential oils on fungal sporulation the method described by Vipotnik et al. (2017) was used with some modifications. The mycotoxigenic fungi were inoculated on PDA medium with 5 µL of a spore suspension adjusted to 10^6 spores/mL in the center of the plate and incubated in the dark at 25°Cfor 5 day.

Isolation of fungal RNA and analysis by quantitative real-time PCR (qRT-PCR)

Mycelium of Petri dish along with the 5 day and 8 days at 25 °C were peeled off from medium supplemented with 0.5 µL of each essential oils finely grinded and grinded using GenoGrinder (SpexSamplePrep, NJ, USA) with steel grinding balls. Total RNA purification was performed through NucleoSpin RNA (Macherey-Nagel, GmbH & Co. KG, Germany), following the manufacturer's instructions. Determination of RNA concentration was measured using the absorbance at 260 nm. All samples were adjusted at 100 ng/µL and first- strand cDNA synthesis reaction was carried out using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Finally, resulting cDNA was diluted with water at 1:20. Gene specific primers used are listed in Table 2.
LightCycler 480 (Roche Diagnostics, Basel, Switzer- land) instrument was used for all qPCR runs. The first step was a pre-incubation at 95 °C for 3 min followed by 45 amplification cycles (95 °C for 10 s, annealing at 68 °C or 64 °C (*aflD* or the others respectively) for 10 s), and a final extending step for 10 s at 72 °C., for melt curve analysis the parameters used are: 95 °C for 5 s, 65 °C for 1 min and up to 97 °C before cooling down to 40 °C. Housekeeping gene β-tubulin was used for data normalization. Relative gene expression ratio of both groups (control and treatments) was determined using the Advanced Relative Quantification LightCycler® 480

Software considering the real-time PCRs efficiencies of the housekeeping gene and the targeted genes (Table 2).

Table 2 List of primers

Target gene	Primer	Sequence (5′ -3′)	Efficiency	References
aflP	*aflP*-F	TGTGTCGAGTGATGTGGGACTAG	2	Hua et al. (2014)
	aflP- R	GCCACCCAGCTCAACCTACA		
aflD	*aflD* -F	CACCATCACCAACATGCAC	2	Wilkinson et al. (2007)
	aflD -R	CTGGATCGATGATGAAGGC		
β tubulin	β tubulin-F	GTTGACCCAGCAGATGTTCGA	1.84	Hua et al. (2014)
	β tubulin-R	TGGAGGTGGAGTTTCCAATGA		
fum 1	*fum 1*-F	ACACCAAAGCCTCTACAGGTGA	2	Visentin et al. (2012)
	fum 1-R	AGGTATCGGGCACCGCT		
fum 8	*fum 8*-F	AGCACAGACGGCGGAGAAGTT	1.81	Visentin et al. (2012)
	fum 8-R	TGAGTTGTCGCTCGCTTGTTG		
β tubulin	β tubulin- F	CAGCGTTCCTGAGTTGACCCAACAG	2	Choi and Shim (2008)
	β tubulin- R	CTGGACGTTGCGCATCTGATCCTCG		

Statistical analysis

All experiments were carried out in 3 replicates. All statistical tests were performed using R software (Rstudio), version 3.6.3. for the test of the effect of essential oils on mycotoxin production and fungal growth, mycelium area evolution of each pathogen in confrontation with each essential oils and concentration of essential oils were monitored overtime (in the day 3, 6, 9 and 12) and measured in cm^2 by image analysis using ImageJ software. For all samples, mycotoxin extraction, detection and quantification were achieved in the same days, for the statistical analysis is in specific point (day 9 for the growth and for mycotoxin production the day in which the strains produce the maximum of mycotoxin).

All experiments were carried out in triplicates. All statistical tests were performed using R software (RStudio), version 3.6.3. Data regarding the sporulation, growth, mycotoxin productions and gene expressions were analyzed through a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$).

Results

Volatile composition of essential oils

The GC/MS analysis of the EOs allowed the identification of the main volatile compounds of the 3 essential oils: (1) *C. schoenanthus* EO, (2) *C. nardus* EO and (3) *E. camaldulensis* EO studied (Table 3).

The main components of *C. schoenanthus EO* (extracted from the leaf part) were as follow: D-limonene (19.60%), p-cymene (11.01%) and B-phellendrene (7%).

C. nardus EO (extracted from the leaf part) was characterized by the presence of high percentages of geraniol (28.16%), citronellol (10%) and caryophyllene-(I1) (6.68%).

E. camaldulensis EO major compounds were γ-terpinene (32.70%), α-pinene (19.22%) and o-Cymene (4.4%).

Table 3 The main volatile compounds detected in essential oils.

	Percentage (%)		
Constituant	*C. schoenanthus*	*C. nardus*	*E. camaldulensis*
α-Pinene	-	-	**19.2±4.9**
α-Phellandrene	6.4±0.8	-	2.2±2.2
(+)-4-Carene	2.8±0.4	-	-
D-Limonene/limonene	**19.6±4.7**	-	-
beta.-Phellandrene	**7.0±1.5**	-	-
p-cymene	**11.0±0.1**	-	-
γ-Terpinene	-	-	**32.8±3.3**
o-Cymene	-	-	**4.4±0.2**
Caryophyllene-(I1)	-	**6.7±0.8**	
Terpinen-4-ol	-	-	3.1±0.5
Caryophyllene	5.9±1.7	-	-
Isopinocarveol	-	-	2.2±0.3
Citronellyl	-	4.1±0.4	-
α-Muurolene	-	2.6±0.3	-
γ-Muurolene	3.4±0.5	-	-
Citronellol	-	**10.0±1.1**	-
Geraniol	-	**28.2±2.5**	-
Thymol	3.0±0.1	-	-
Other identified compounds	28.5	45.5	36.1
Total	**87.6**	**97.1**	**100**

Results correspond to the mean ± standard deviation.

Effects of essential oils on fungal growth

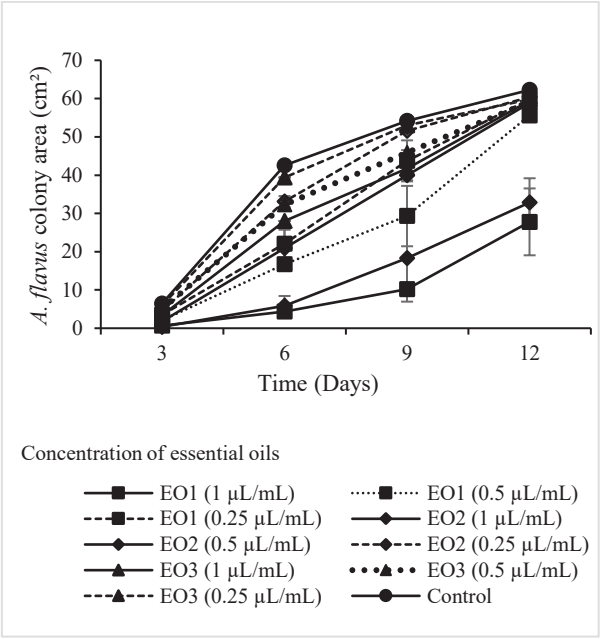
The growth curves obtained for the 3 strains *A. flavus*, *A. carbonarius* and *F. verticillioides*, in PDA medium supplemented with different concentrations (0, 0.25, 0.5 and 1 µL/mL) of *C. schoenanthus, C. nardus* and *E. camaldulensis* EOs, using agar diffusion method, are shown in Fig. 1.

The 3 EOs delayed fungal growth with respect to the control. *C. schoenanthus* and *C. nardus* EOs appeared to be more efficient than *E. camaldulensis* EO.

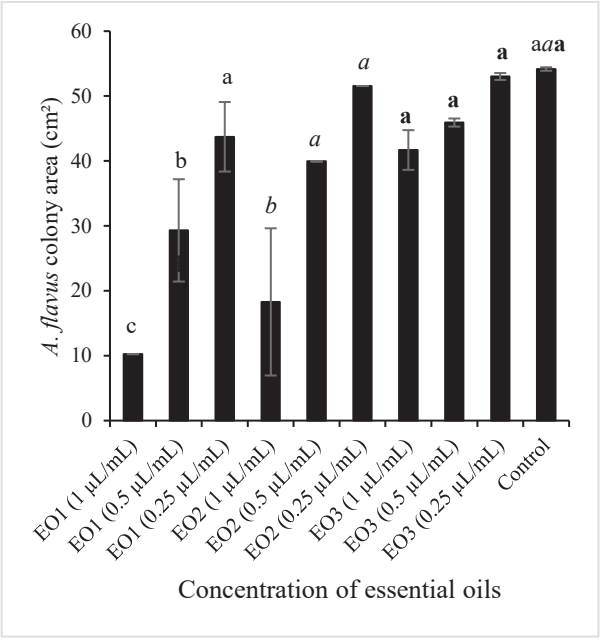
The statistical analysis regarding the influence of the EOs on the growth of the 3 strains on day 9 (Fig. 1b,1d and 1f) showed a strong effect of EOs concentrations. The highest antifungal

effect of *Cymbopogon* EOs was obtained with 1 µL/mL for the 3 fungal strains. Only the growth of *F. verticillioides* was significantly impacted by *E. camaldulensis* EO.

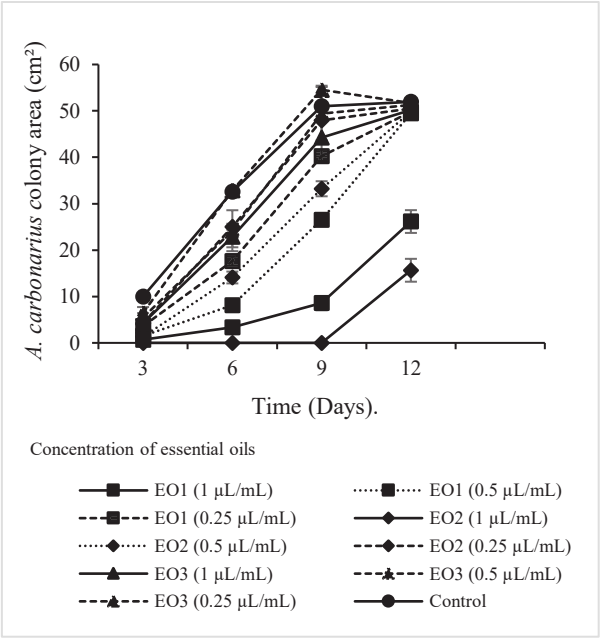
(a)



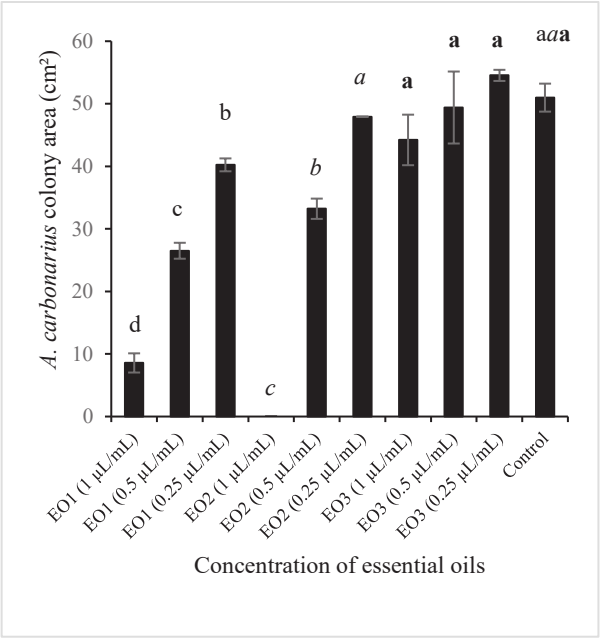
(b)



(c)



(d)



(e) (f)

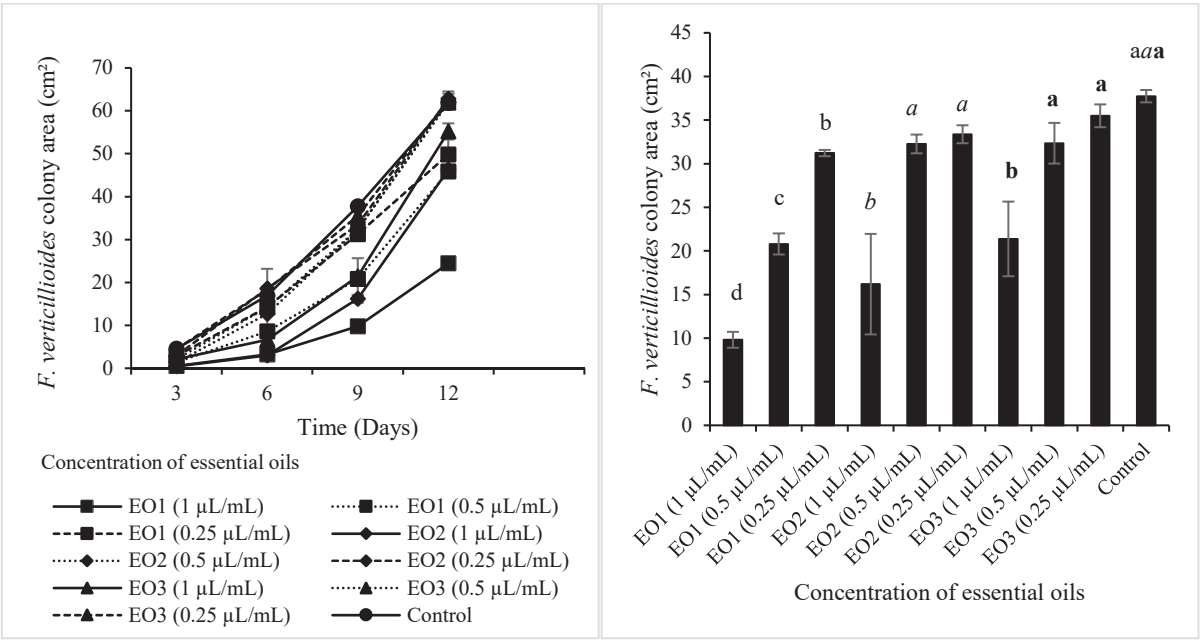
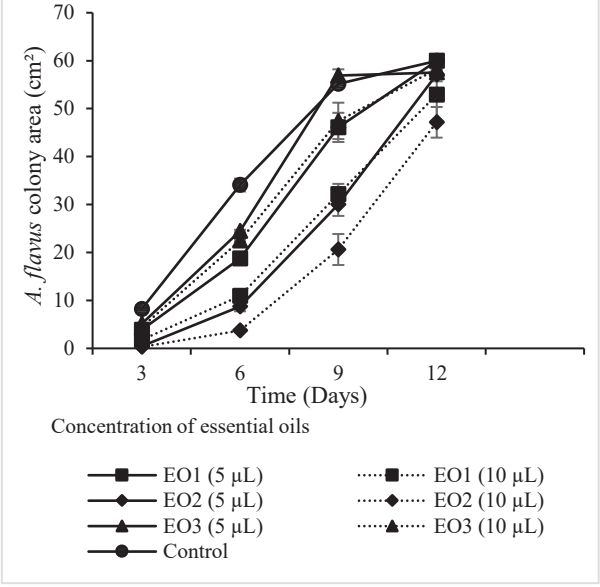


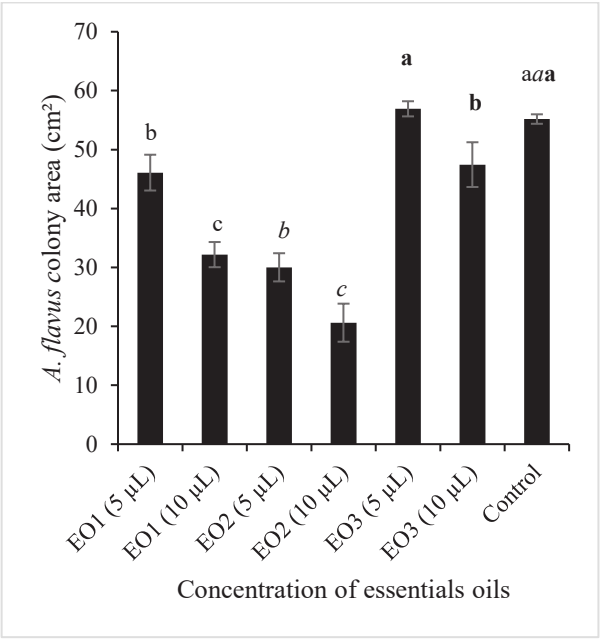
Fig. 1 Effect of different concentrations of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) essential oils on fungal colony surface (cm²) in PDA medium at 25°C (Agar diffusion method). (a) Growth kinetic of *A. flavus* incubated for 12 days. (b) Growth of *A. flavus* on day 9. (c) Growth kinetic of *A. carbonarius* incubated for 12 days. (d) Growth of *A. carbonarius* on day 9. (e) Growth kinetic of *F. verticillioides* incubated for 12 days. (f) Growth of *F. verticillioides* on day 9. Differences between control and tested essential oils were determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

The results related to the inhibition of fungal growth resulting from the exposure of fungi to EO vapors are given in Fig. 2. As observed in the agar diffusion method, the inhibition of growth increased with increasing EO concentrations. On day 9, the growth of the 3 strains was more impacted by *C. schoenanthus* and *C. nardus* EOs than by *E. camaldulensis* EO.

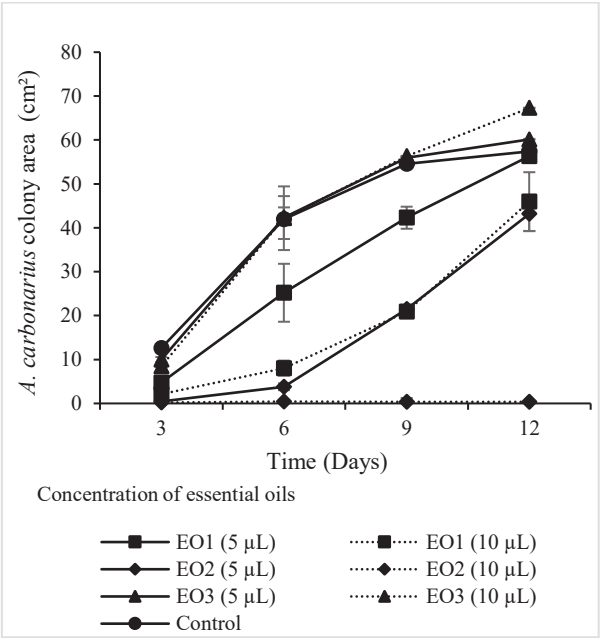
(a)



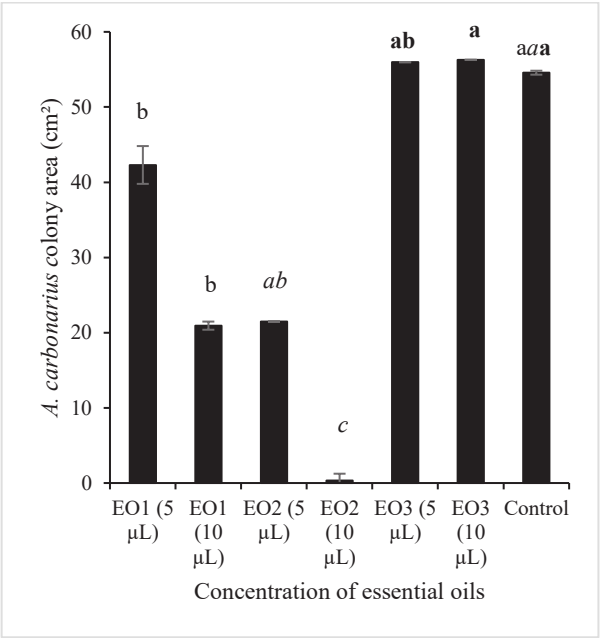
(b)



(c)



(d)



(e) (f)

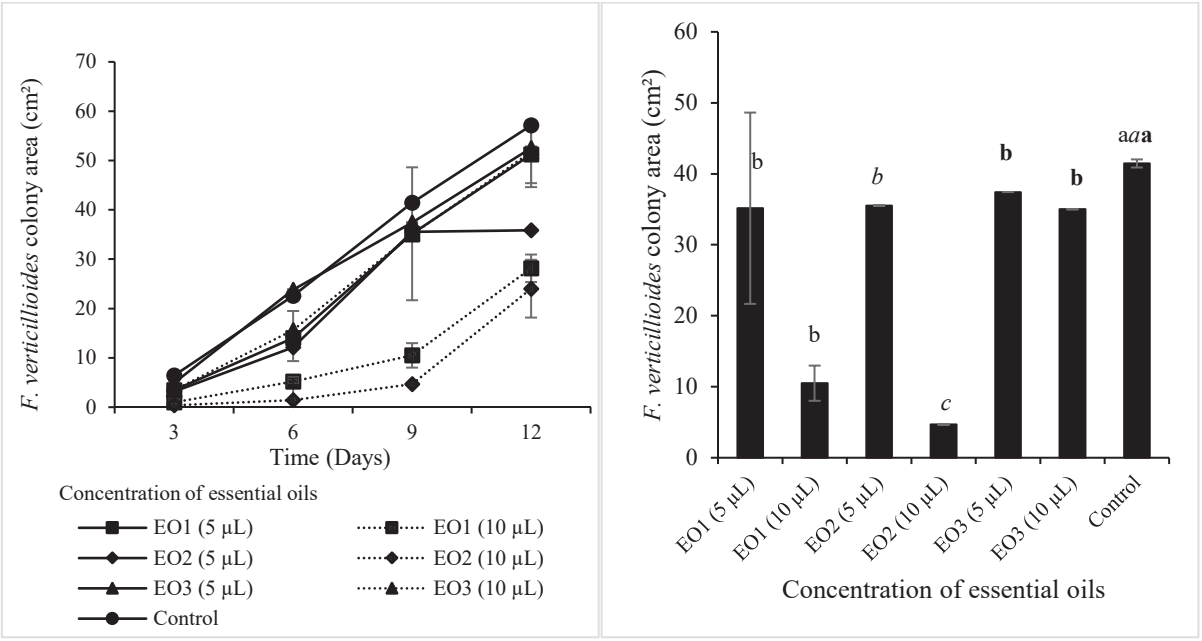
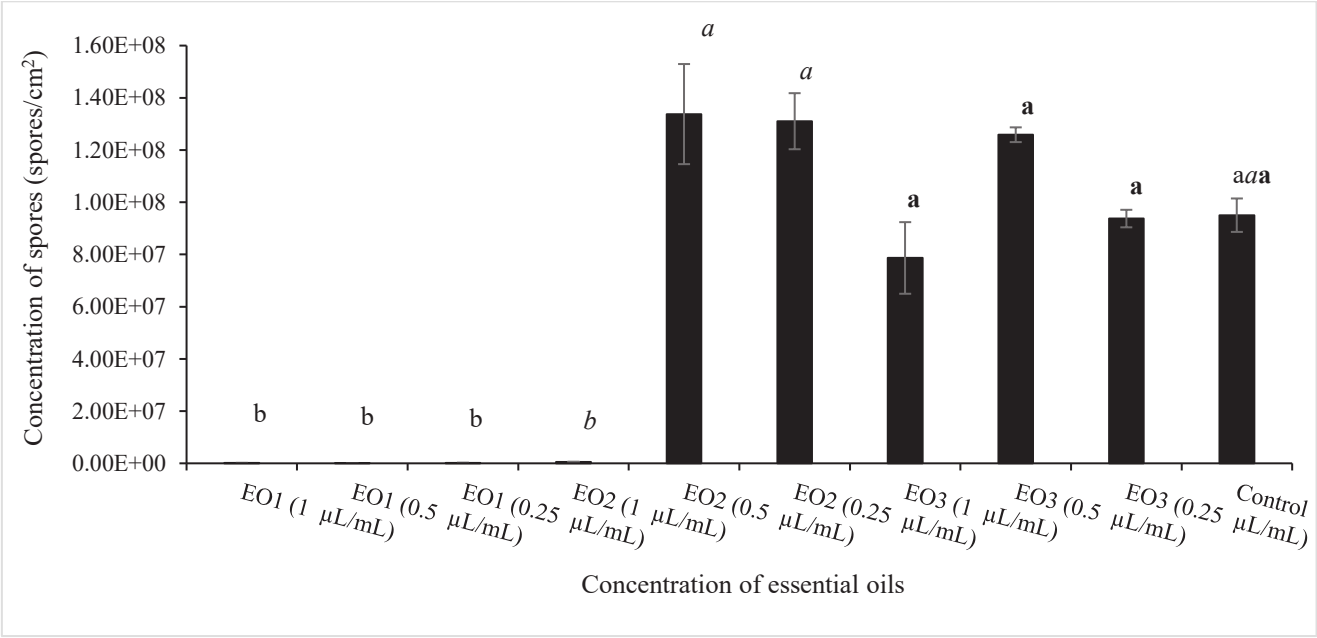


Fig. 2 Effect of different concentrations of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) volatile compounds on fungal colony surface (cm²) in PDA medium at 25°C (Disc volatilization method). (a) Growth kinetic of *A. flavus* incubated for 12 days. (b) Growth of *A. flavus* on day 9. (c) Growth kinetic of *A. carbonarius* incubated for 12 days. (d) Growth of *A. carbonarius* on day 9. (e) Growth kinetic of *F. verticillioides* incubated for 12 days. (f) Growth of *F. verticillioides* on day 9. Differences between control and tested essential oils were determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

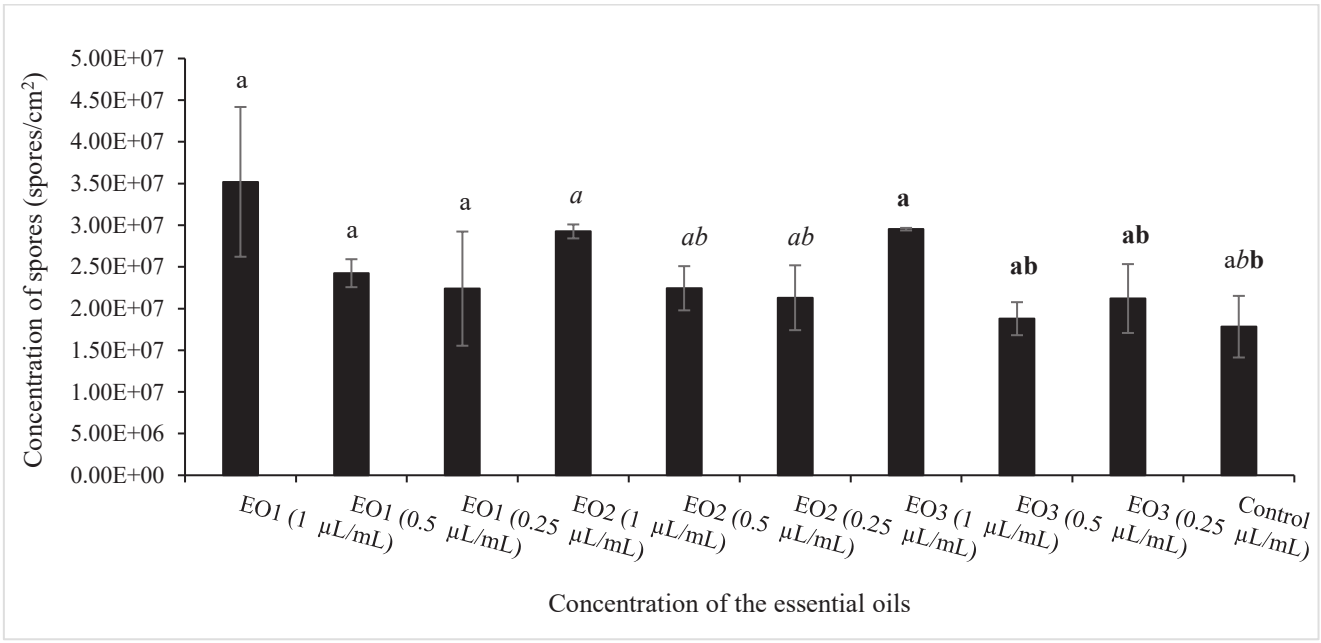
Effects of essential oils on fungal sporulation

The sporulation results obtained for the 3 strains *A. flavus*, *A. carbonarius* and *F. verticillioides*, in PDA medium supplemented with different concentrations (0, 0.25, 0.5 and 1 µL/mL) of *C. schoenanthus, C. nardus* and *E. camaldulensis* EOs, are shown in Fig. 3. It appeared that only the sporulation of *A. flavus* was negatively affected by: (1) *C. schoenanthus* EO at all concentrations tested and (2) *C. nardus* EO at 1 µL/mL. To a lesser extent sporulation of *F. verticillioides* was impacted by 1 µL/mL of *C. nardus* EO. Regarding *A. carbonarius*, stimulation of sporulation was observed, mainly when using the highest concentration of EOs.

(a)



(b)



(c)

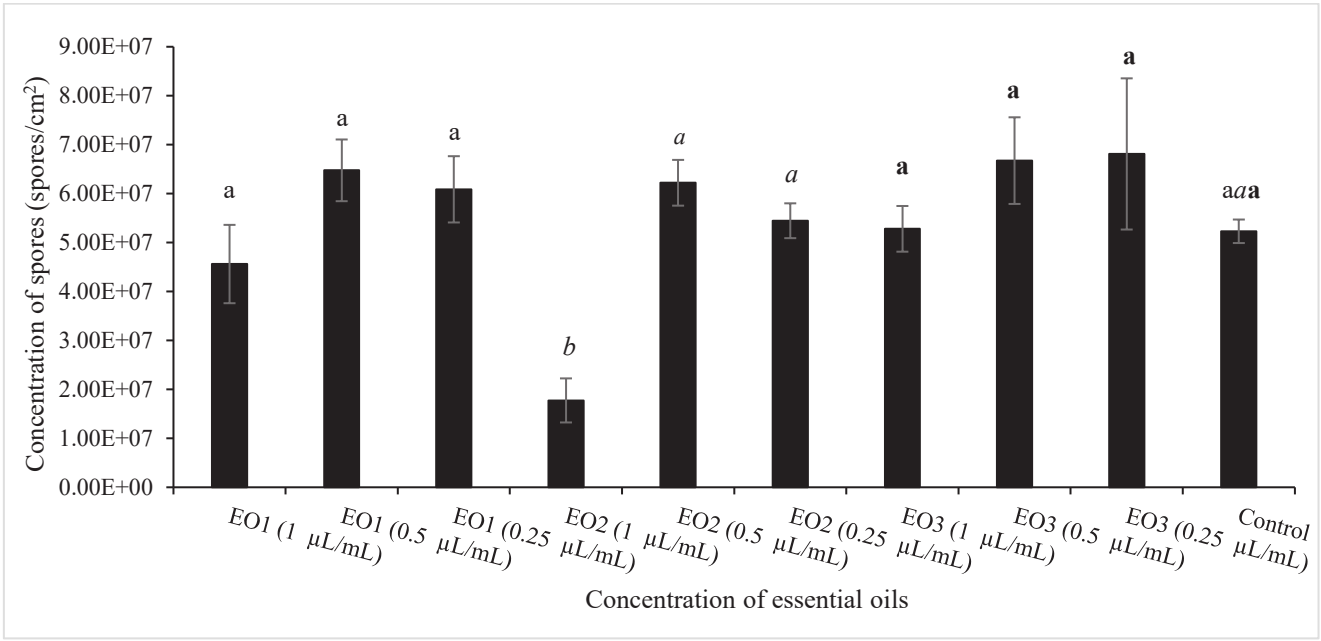


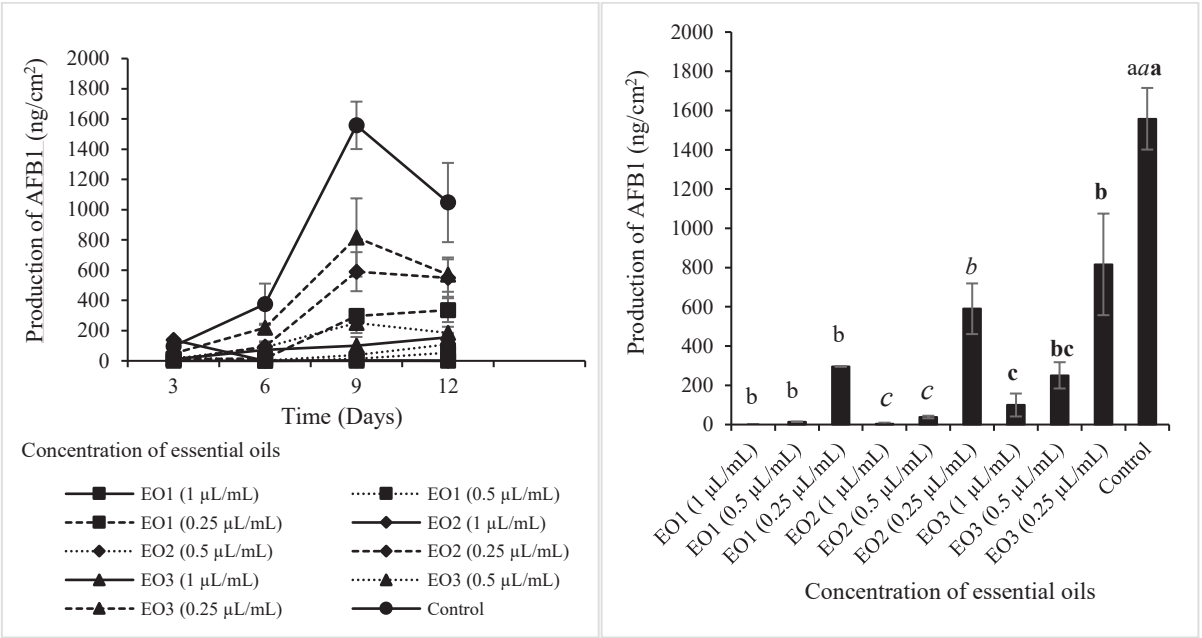
Fig. 3 Effects of different concentration of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) essential oils on spore production after 5 days in PDA medium at 25 °C. (a) for *A. flavus*. (b) for *A. carbonarius.* (c) for *F. verticillioides*. Differences between control and tested essential oils were determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

Effects of essential oils on mycotoxin content

The amounts of aflatoxin B1 (AFB1), ochratoxin A (OTA) and fumonisin B1 (FB1) produced by *A. flavus*, *A. carbonarius* and *F. verticillioides*, respectively, after 12 days of incubation in presence of the 3 EOs using agar diffusion method were determined. Results are shown in Fig. 4.

The statistical analysis regarding AFB1 on day 9 in the presence of the 3 EOs showed significant differences between control and treatments. The *C. schoenanthus* EO was able to strongly reduce the content of AFB1 under the 3 concentrations used. Significant reductions were also achieved for both *C. nardus* and *E. camaldulensis* EOs, especially with the concentrations of 1 and 0.5 µL/mL but *E. camaldulensis* EO was less efficient than *C. schoenanthus* and *C. nardus* in the reduction of AFB1 content. Regarding OTA, the content was significantly reduced by *C. schoenanthus* EO, a little less by *C. nardus* EO and *E. camaldulensis* EO was the least effective. In the case of FB1, the results were close to those obtained with AFB1 although the inhibition was less important at lower concentrations. The best inhibition levels were detected using *Cymbopogon* EOs at the concentration of 1 µL/mL, which was more efficient than *E. camaldulensis* EO.

(a) (b)



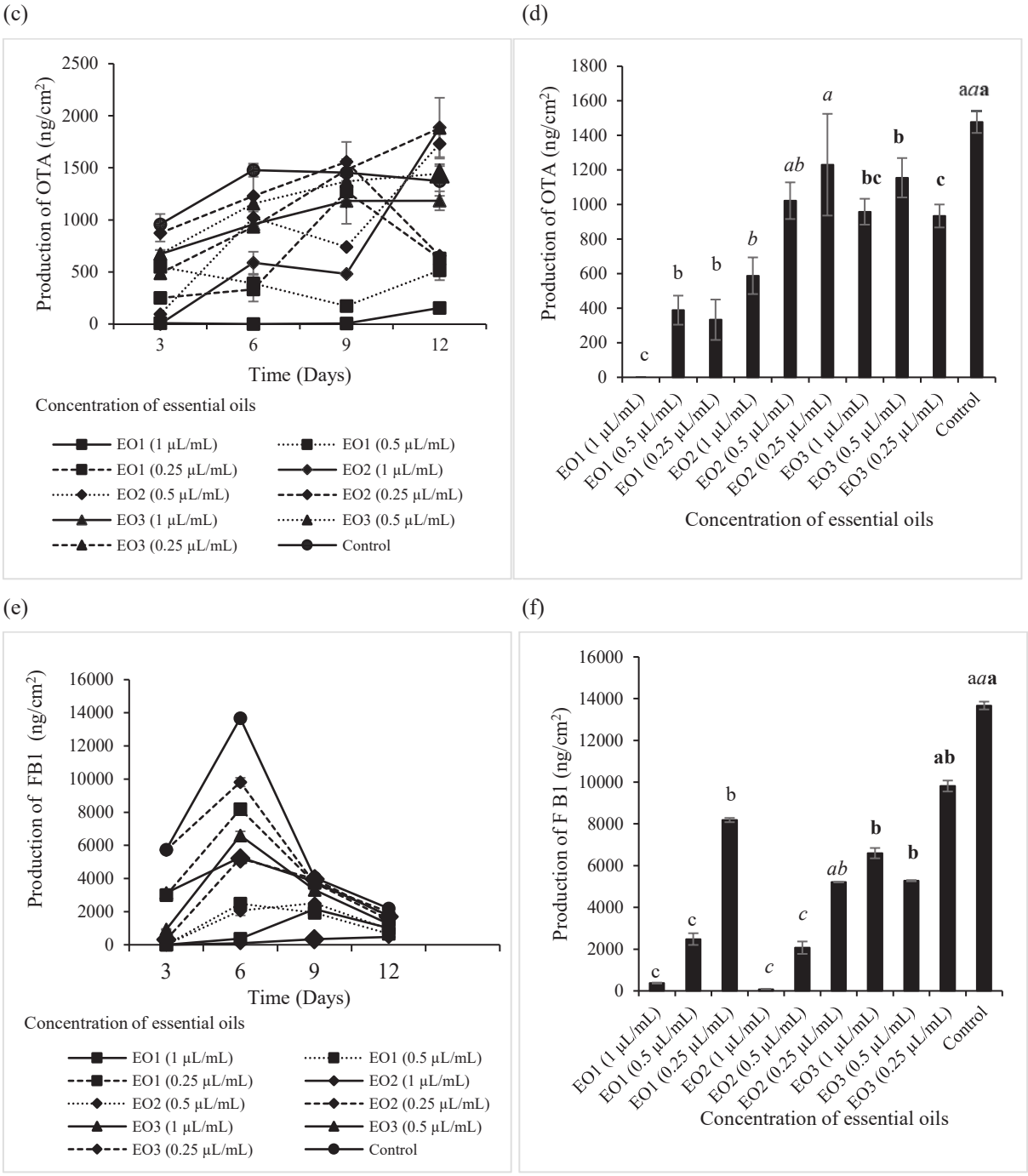
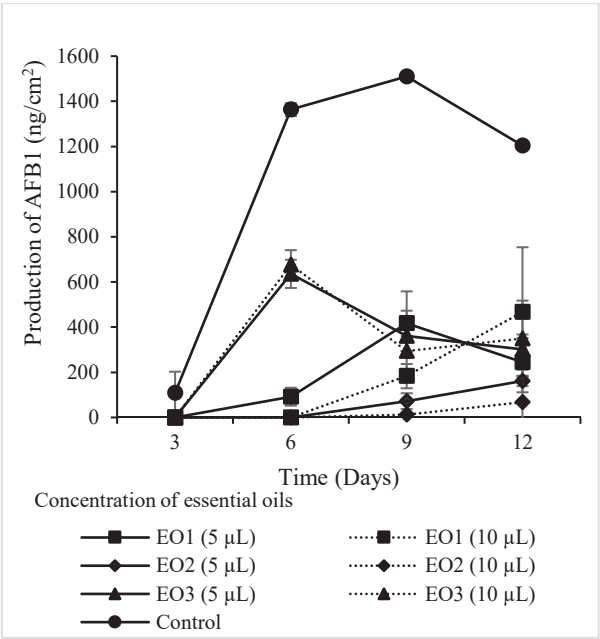


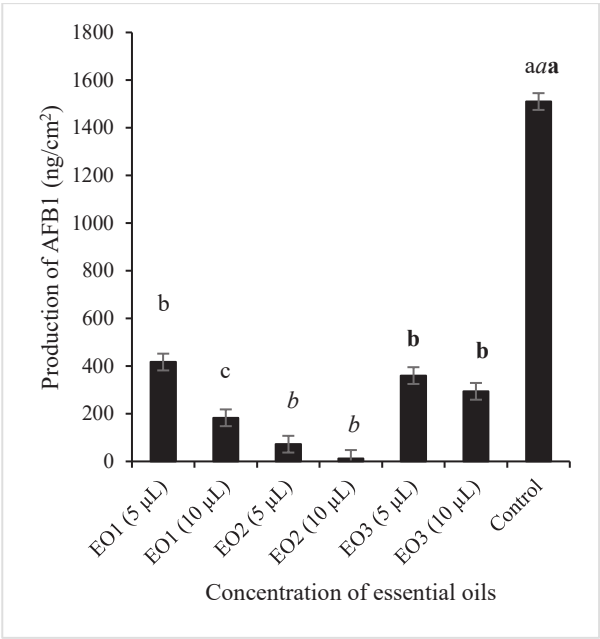
Fig. 4 Effects of different concentrations of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) essential oils on mycotoxin production in PDA medium at 25 °C (Agar diffusion method). (a) Specific production of AFB1 during 12 days. (b) Specific production of AFB1 on day 9. (c) Specific production of OTA during 12 days. (d) Specific production of OTA on day 6. (e) Specific production of FB1 during 12 days. (f) Specific production of FB1 on day 6. Differences between control and tested essential oils were determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

The results of the effect of 2 concentrations of EO vapors on the mycotoxin contents are presented on Fig. 5. The effectiveness of the EOs vapors changed depending on the essential oil used and the tested strain. While *C. nardus* EO was strongly efficient in reducing AFB1 and FB1 contents on day 9, better results were obtained with *C. schoenanthus* EO on OTA. Although appearing less effective, *E. camaldulensis* EO exhibited significant effects on the 3 mycotoxins and more particularly on the FB1 content.

(a)



(b)



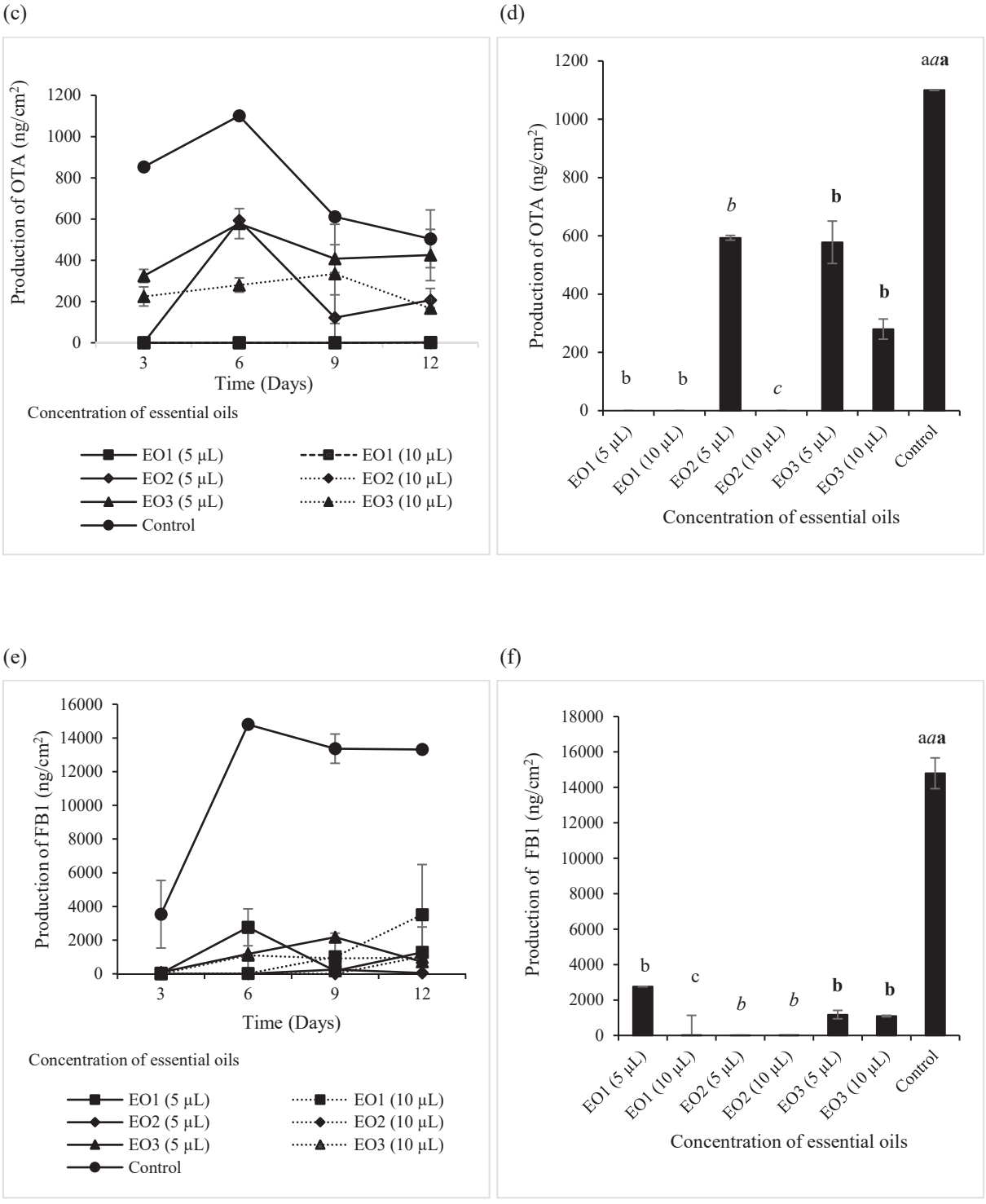


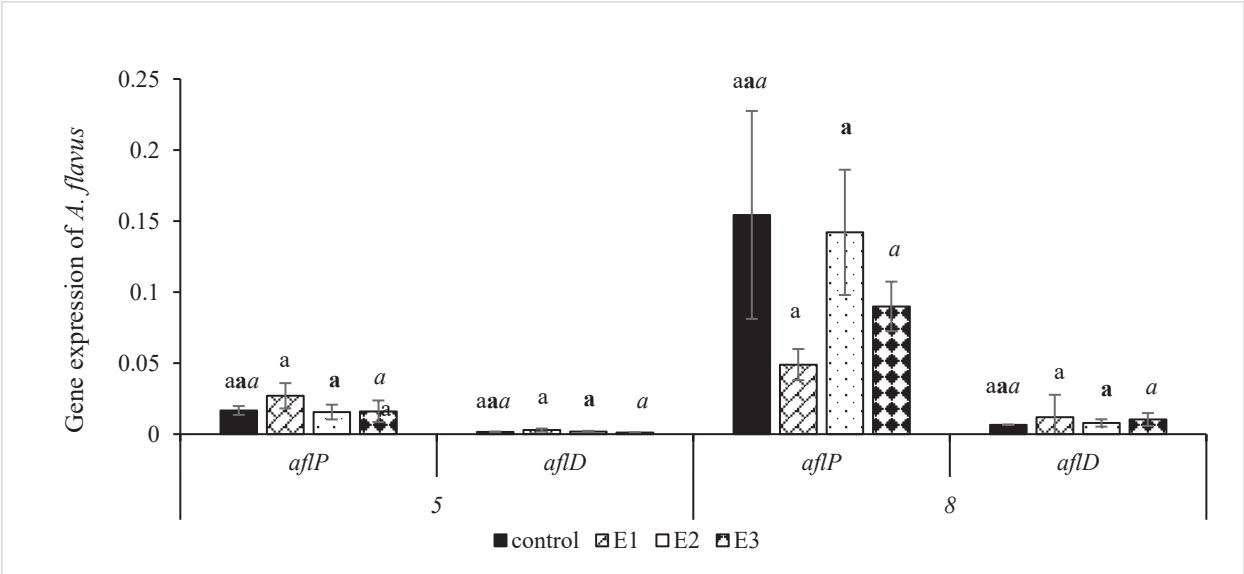
Fig. 5 Effects of different concentrations of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) volatile compounds on mycotoxin production in PDA medium at 25 °C (Disc volatilization method). (a) Specific production of AFB1 during 12 days. (b) Specific production of AFB1 on day 9. (c) Specific production of OTA during 12 days. (d) Specific production of OTA on day 6. (e) Specific production of FB1 during 12 days. (f) Specific production of FB1 on day 6. Differences between control and tested essential oils were

determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

Effects of essential oils on AFB1 and FB1 biosynthesis pathways

In *A. flavus*, AFB1 biosynthesis is the result of an enzymatic cascade involving 27 genes grouped in a cluster. The impact of the essential oils on two of the major AFB1 biosynthetic genes i.e. *aflD* and *aflP* is presented in Fig. 6a. Following EOs exposure, both genes expression did not present any significant changes when compared to control at both day 5 and 8. The fumonisins biosynthetic pathway in *F. verticillioides* was affected following exposure to EOs of *C. schoenanthus* EO and *C. nardus* EO (Fig 6b). *Fum1* expression was up-regulated after 5 and 8 days of exposure of the fungus to *C. nardus* EO. In contrast, it was repressed at day 8 by exposure to *C. schoenanthus* EO. Inversely, *fum8* expression appeared to be induced after 8 days of exposure of *F. verticillioides* to EO1. The EO of *E. camaldulensis* did not seem to impact genes expression of the two mycotoxins biosynthetic pathways targeted in this study.

(a)



(b)

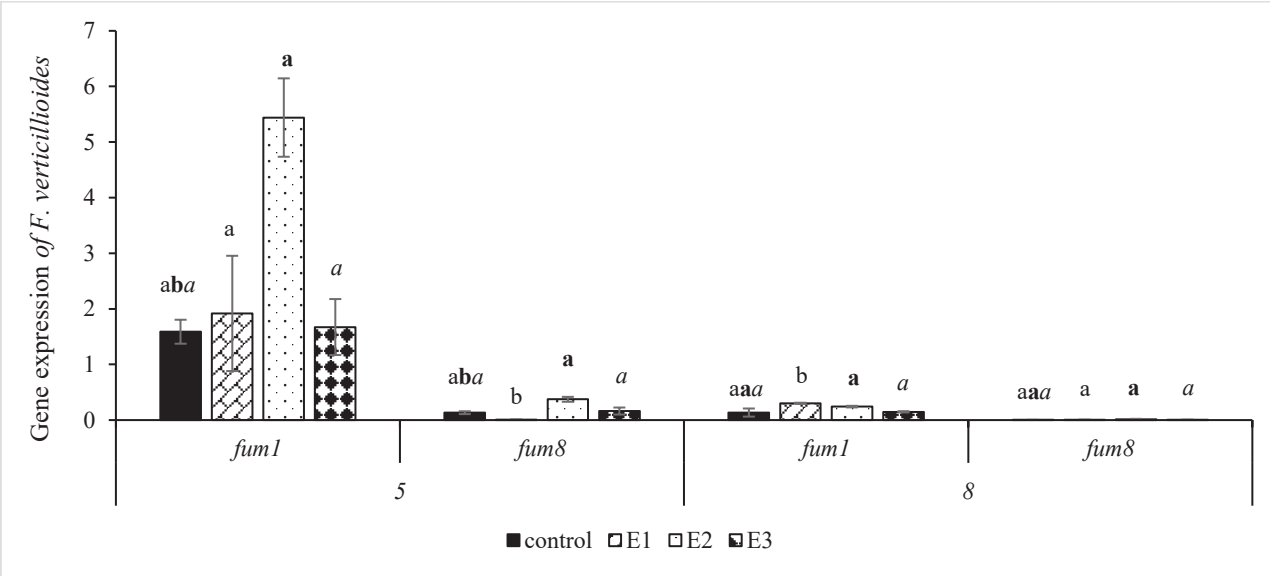


Fig. 6 Comparative effects of *C. schoenanthus* (EO1), *C. nardus* (EO2) and *E. camaldulensis* (EO3) essential oils on gene expression. (a) Expression of genes (*aflD* and *aflP*) involved in aflatoxin B1 biosynthesis in *A. flavus* grown in contact with 0.5 µL/mL of essentials oils and incubated at 25 °C in PDA medium for 5 or 8 days. (b) Expression of genes (*fum1* and *fum2*) involved in fumonisin B1 biosynthesis in *F. verticillioides* grown in contact with 0.5 µL/mL of essentials oils and incubated at 25 °C in PDA medium for 5 or 8 days. Differences between control and tested essential oils were determined separately for each EOs according to ANOVA and Tukey's multiple comparison tests. For each EOs, bars with different letters indicate significant differences ($p < 0.05$).

Discussion

The qualitative GC/MS analysis of the 3 essential oils made it possible to highlight composition differences and notably regarding the principal volatile compounds. The main components of *C. schoenanthus* EO were D-limonene (19.60%), p-cymene (11.01%) and B-phellendrene (7%), which is consistent with the results obtained by Aous et al. (2019). *C. nardus* EO was characterized by the presence of high percentages of geraniol (28.16%), citronellol (10%), caryophyllene-(I1) (6.68%). These results are in agreement with the literature with slight percentage differences (Aguiar et al., 2014b; Nakahara et al., 2003). *E. camaldulensis* EO major compounds were γ-terpinene (32.70%), α-pinene (19.22%) and o-Cymene (4.4%). It can be noted that 1,8-ceneol (Eucalyptol) was detected in small quantities contrary to what was observed by Aleksic Sabo and Knezevic (2019b) as well as Gakuubi (2016). The variation in the chemical composition of the essential oils between the results of this study and the results obtained in the literature may be due to various factors. Among them: (1) exogenous factors such as the nature and components of the soil, temperature, altitude; (2) endogenous factors such as the genetic heritage of individuals (Chantal et al., 2016; Tanoh et al., 2020; Turek & Stintzing, 2013), and (3) the extraction technique or method used to obtained the essential oils. Ecological conditions in different countries can influence the chemical profile of plant materials. Some compounds can be accumulated at a particular time in response to environmental conditions. EOs collected in different regions at different seasons have a different chemical composition and can therefore exhibit different biological activities (Basak & Guha, 2018; Chantal et al., 2016; Sadeh et al., 2019). For example, the citronellal content can vary among the cultivated varieties of citronella grass. Indeed, citronellal was found to account for 40.5-60.7% of the essential oil of Maha Pengiri (Sri Lanka and Indonesia) while only 17.2-33.2% was found in the oil of *C. nardus* var. *confertiflorus* (India) (Nakahara et al 2003).

Analysis of the antifungal activity on both diffusion method and disc volatilization method revealed a high sensitivity of all strains to the essential oils. This activity was higher for the highest EO concentrations for all strains in the diffusion method (1µL/mL) and the disk volatilization method (10 µL). However, *E. camaldulensis* EO appeared to be significantly less effective than the other two. A study conducted by Achar et al. (2020) also showed that the antifungal potential of *Cymbopogon winterianus* and *Cymbopogon citratus* EOs were greater than *Eucalyptus globulus* EO. The differences in activities between EOs could be explained,

according to Tantaoui-Elaraki (1993), by the fact that EOs antimicrobial activities can be classified in a decreasing order, according to the nature of their major compound. Inouye et al. (2006) studied the vapor activity of 72 essential oils against *Trichophyton mentagrophytes* and demonstrated that essential oils with phenol as the major component displayed the most activity. In decreasing order of activity, from most to least potent, they suggested that the order of potency of essential oil vapors can be determined as those rich in phenol > aldehyde > alcohol > ketone > ester (= ether/oxide) > hydrocarbon. The antifungal power of the EOs of *Cymbopogon* species can be explained by the presence of D-limonene and geraniol in *C. schoenanthus and C. nardus* respectively. According to Tang et al. (2018), geraniol at a concentration of 0.6 µL/mL, exerted a strong inhibition of mycelial growth of *A. flavus* and *A. ochraceus*, The inhibition rate of geraniol against *A. flavus* and *A. ochraceus* was 98.4% between 0.5 and 0.6 µL/mL. D-limonene was reported to exhibit promising antifungal activity against *Aspergillus niger, Fusarium oxysporum, Phytophthora digitatum, F. verticillioides, R. solani, and S. sclerotiorum* (Abo Elgat et al., 2020). A study performed by Omran et al. (2011) showed that D-limonene has an inhibitory effect from on *A. niger* growth.

It was also noticed that there were differences in sensitivity between species belonging to the same genus (*A. flavus* and *A. carbonarius*) and between the genera *Aspergillus* and *Fusarium* (*F. verticillioides*). The mechanisms of action of EOs by which mycelial growth can be reduced or totally inhibited are various. The lipophilic property of the EOs helps them in crossing the fungal cell membrane and inter-acting with the enzymes and proteins of the membrane. Most of the mechanisms involved modify the fungal growth rate and extended the lag phase, interact with the cell membrane disrupting cell permeability, and damage the enzymatic cell system. This facts result in irreversible harmful morphological and ultra-structural changes, reduction of mycelial growth, spore germination inhibition, disruption of the oxidative balance and the electron transport chain, inhibition of energy release and reduction in DNA formation binding (Chaudhari et al., 2019; Chelaghema et al., 2021).

The sporulation of the 3 fungal strains was variously affected by the EOs. Indeed, the sporulation of *A. flavus* was very significantly decreased by *Cymbopogon* EOs by opposition to *A. carbonarius* sporulation that appeared stimulated. These results are partially in agreement with Císarová et al. (2020) report that showed a significant negative effect of different EOs, including *Cymbopogon citratus* EO, on 4 different strains of *Aspergillus*.

The inhibition of the production of mycotoxins was analyzed in the presence of 3 concentration (0.25, 0.5 and 1 µL/mL) of each essential oils with agar diffusion method and 2 concentrations

(5 and 10 µL/disc) with disc volatilization method. In the case of direct contact, the AFB1 content was most impacted by the EOs. For all mycotoxins, *Cymbopogon* EOs were more effective although *Eucalyptus* EO showed a significant effect on the AFB1, OTA and FB1. The good results obtained for AFB1with *C. nardus* EO were consistent with previous research from Mahmoud (1994) who showed the effectiveness of geraniol in limiting contamination by this toxin.

Kumar et al. (2020) investigated the *in vitro* and *in vivo* efficacy of *Artemisia nilagirica* EO for shelf-life enhancement of stored millets by preventing fungal infestation and AFB1 contamination. During in vitro testing, the growth of toxigenic *A. flavus* and AFB1 secretion was completely inhibited at 1.4 and 1.0 µL/mL, respectively. The EO exhibited strong antioxidant activity, which significantly inhibited the AFB1 biosynthesis by scavenging the free radicals and prevented the risk of nutritional quality deterioration by oxidation of food components.

The mycotoxins content was strongly influenced by EOs volatile compounds and, as in the agar diffusion method, *Eucalyptus* EO was the less effective. However, in this case, it was the FB1 that was most affected.

The precise modes of action of EOs on mycotoxinogenesis are poorly understood, but various mechanisms have been proposed, including inhibition of production through action on synthetic genes (Chaudhari et al., 2019; Chelaghema et al., 2021; Mirza Alizadeh et al., 2021; Perczak et al., 2020; L. Wang et al., 2019)

To improve the understanding of how these EOs act on mycotoxinogenesis, their effects on the transcriptional level of genes involved in the inhibition of the biosynthesis of AFB1 and FB1 were assessed. Expression of genes involved in the biosynthetic pathways of AFB1 and FB1 were analyzed by real-time RT-PCR (genes *aflD* and *aflP* for AFB1 and *fum1* and *fum8* genes for FB1). None of the genes studied were significantly repressed in *A. flavus* exposed to the 3 EOs. These results do not agree with those obtained by Oliveira et al. (2020) who used *Thymus vulgaris* EO for *in vitro* inhibition of *A. flavus* and AFB1 secretion. The EO downregulated (49.4%) the expression of *laeA* gene implicated in aflatoxin biosynthesis. El Khoury et al. (2016) suggested the active participation of rosemary, fennel, anise, chamomile, cardamom, and celery EOs in reducing the expression of *acOTApks, laeA, acOTAnrps, acpks,* and *veA* genes, which are involved in ochratoxin A biosynthesis in *A. carbonarius*. The authors reported the effects on ochratoxigenic gene expression at 1.0 µL/mL of different test EOs, and variability in their downregulating gene expression was affected by nature and chemical constituents of

EOs. Kim et al. (2018) reported that the downregulation of norsolorinic acid (NOR) reductase and versicolorin B (VERB) synthase gene (*AflE* and *AflL*, respectively) upon 1, 8 cineole treatment as the major cause responsible for the inhibition of AFB1 biosynthesis. Tian et al. (2018) reported the efficacy of 3 important monoterpenes, namely p-cymene, carvocrol and thymol, in inhibition of aflatoxin biosynthesis and reported the inhibition of fungal melanins very much similar to norsolorinic acid, which is the first important metabolite of AF biosynthesis, as the probable cause for aflatoxin inhibition. Moreover, the action of EOs on the biosynthetic pathway of fumonisins is contrasted. Finally, in order to obtain further explanations on how essential oils limit the accumulation of mycotoxins, it would be wise to study the transcriptome as a whole by a non-targeted experimental approach such as RNAseq over several days of exposure of the fungus to the essential oil.

Conclusion

The 3 essential oils provide inhibitory effects on growth and mycotoxin production of *A. flavus, A. carbonarius* and *F. verticillioides* (AFB1, OTA and FB1 respectively) grown on PDA medium. It is noteworthy to mention that *E. camaldulensis* EO was able to inhibit the production of mycotoxins with little or no significant effect on fungal development. Indeed, this suggest the possibility to limit the contamination by mycotoxins without modifying the microbiota during field use, and thus avoiding the emergence of new potentially dangerous species. Essential oils could also be used in bioactive packaging to control fungal spoilage in food.

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Authors' contributions All authors have read and agree to the published version of the manuscript. Asma Chelaghema, Angélique Fontana and Caroline Strub designed the study. Noël Durand performed mycotoxin analysis via HPLC. Adrien Servent performed essential oils analysis via GC/MS. Myriam Mamouni participated in manipulation of qPCR. Asma Chelaghema carried out the entire range of manipulations and experimental approaches and analyzed the data. Asma Chelaghema, Caroline Strub, and Angélique Fontana interpreted the data. Asma Chelaghema wrote the original draft. Angélique Fontana, Caroline Strub, Patrick Poucheret and Sabine Schorr-Galindo carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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Availability of materials Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethic approval Not applicable.

Consent to participate Not applicable.

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Conclusion

Les trois huiles essentielles ont des effets inhibiteurs sur la croissance et la production de mycotoxines par *A. flavus*, *A. carbonarius* et *F. verticillioides* (AFB1, OTA et FB1 respectivement) cultivés sur milieu PDA dans les deux méthodes testées qui sont la méthode de diffusion directe et la méthode de diffusion sur disque. Néanmoins, il est vraiment intéressant de noter que l'E. camaldulensis est capable d'inhiber la production de mycotoxines sans avoir inhibé ou peu inhibé la croissance fongique. Cela permet de supposer que cette huile essentielle permettrait de maîtriser le risque mycotoxinogène sans altérer le microbiote associé à la denrée et donc éviter l'apparition de nouvelles espèces potentiellement dangereuses. Les trois huiles essentielles ont des effets contrastés sur l'expression des gènes des voies de biosynthèse des différentes mycotoxines étudiée. Il serait donc souhaitable de mener des études plus approfondies et complémentaires afin de déterminer plus précisément leur mode d'action comme par exemple étudier l'impact des HE sur les transcriptomes fongiques de manière non ciblée.

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Conclusion générale

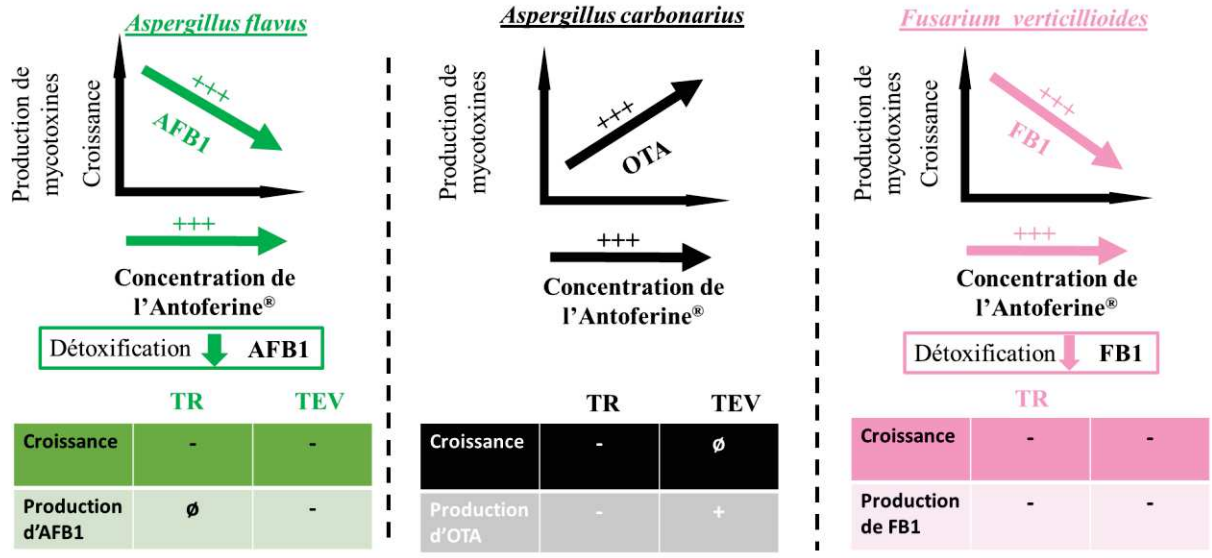
Conclusion générale

L'objectif global de ce travail était de tester les activités antifongique et/ou antimycotoxique des extraits des plantes. Ces travaux s'inscrivent dans un objectif global de la diminution de l'emploi intensif des produits phytosanitaires de synthèse dans la lutte contre les champignons phytopathogènes mycotoxinogènes, et de trouver des solutions alternatives, dites biologiques, en utilisant des substances naturelles aux propriétés antifongiques. Pour cela, trois huiles essentielles *Cymbopogon schoenanthus*, *Cymbopogon nardus* et *Eucalyptus camaldulensis* ont été utilisées. En plus d'utiliser les huiles essentielles, nous avons utilisé un produit de biocontrôle : l'Antoferine®.

La partie bibliographique est divisée en trois parties. La première correspond à une revue bibliographique qui a permis de faire une synthèse des connaissances sur les principaux champignons filamenteux mycotoxinogènes et leurs mycotoxines, les extraits de plantes utilisés pour le biocontrôle de la contamination par les mycotoxines ainsi que les mécanismes d'action impliqués dans l'inhibition de la croissance fongique et/ou de la contamination par les mycotoxines par les extraits de plantes. La seconde partie avait pour objectif de présenter l'Antoferine®, un éco-extrait polyphénolique de la vigne, produit par la société Antofénol et dont les propriétés antifongiques et antimycotoxique ont été étudiées au cours de ce travail de thèse. La dernière partie est consacrée aux huiles essentielles et plus spécifiquement aux huiles essentielles de *C. nardus, C. schoenanthus* et *E. camaldulensis* qui ont également été testées pour leur activités antagonistes sur la croissance fongique et la mycotoxinogenèse.

La partie consacrée aux résultats expérimentaux est organisée en deux articles, le premier avait pour objectif d'étudier l'effet de l'Antoferine® sur trois souches mycotoxinogènes *A. flavus*, *A. carbonarius* et *F. verticillioides*, L'Antoferine® a montré une activité dose-dépendante sur la croissance et la production de toxines, et dans une moindre mesure sur la production de spores, d'*A. flavus* et de *F. verticillioides.* Son effet sur la croissance d'*A. carbonarius* est plus contrasté puisque la croissance n'est que faiblement impactée alors que la production de toxines est stimulée. L'Antoferine® a également montré un potentiel de détoxification de l'aflatoxine B1 produite par *A. flavus*. Au niveau microscopique, en utilisant la microscopie électronique à balayage (MEB) des modifications de la morphologie des cellules fongiques de *A. flavus* cultivé sur PDA avec 20 g/L d'Antoferine® ont été observées par rapport au témoin. En utilisant la microscopie électronique à transmission (MET), les effets de l'Antoferine® ont été observés à

différents niveaux de l'ultrastructure *d'A. flavus*. Les principaux changements comprenaient une fuite du contenu cytoplasmique ainsi qu'un cytoplasme très dense, pourvu de larges vacuoles. Le Trans-resvératrol et la Trans-Ɛ-viniférine, composés majoritaires de l'Antoferine®, ont affecté la croissance et la production de toxines d'A. *flavus, A. carbonarius* et *F. verticillioides* selon les souches et la concentration des composés. Au niveau transcriptomique l'Antoferine® (1 g/L) à moduler les niveaux d'expression de certains gènes impliqués dans les voies de biosynthèse de AFB1 et FB1. Les gènes ciblés étaient *aflD* et *aflP* pour *A. flavus* et *fum1* et *fum8* pour *F. verticillioides.* Les gènes *TBAf* et *TBFv* (β-tubuline) ont été utilisés pour normaliser l'expression des gènes. Après 5 ou 8 jours de croissance en présence d'Antoferine®, aucun changement dans l'expression des gènes *fum1* et *fum8* n'a été observé. Concernant *A. flavus*, En présence de l'Antoferine® *aflP* était sous-exprimé et *aflD* était surexprimé par rapport au contrôle au jour 8.



TR: Trans-resvératrol TEV: Trans-Ɛ-viniférine

Figure 15 : Principaux résultats obtenus après confrontation des souches mycotoxinogènes avec l'Antoferine®

Le second article avait pour objectif d'étudier 3 huiles essentielles contre 3 souches mycotoxinogènes *A. flavus*, *A. carbonarius* et *F. verticillioides*. Les 3 HEs ont eu des effets inhibiteurs sur la croissance et la production de mycotoxines d'*A. flavus*, *A. carbonarius* et *F. verticillioides* (respectivement AFB1, OTA et FB1) cultivés sur milieu PDA en utilisant deux méthodes expérimentales : la méthode de diffusion directe et la méthode de diffusion sur

disque. Il est intéressant de noter que *E. camaldulensis* est capable d'inhiber la production de mycotoxines sans effet significatif ou moindre sur le développement fongique. Pour mieux comprendre comment ces 3 huiles essentielles agissent sur la mycotoxinogenèse, leurs effets sur le niveau transcriptionnel de gènes impliqués dans la biosynthèse d'AFB1 et FB1 ont été évalués. L'expression des gènes impliqués dans les voies de biosynthèse d'AFB1 et FB1 a été analysée par RT-PCR en temps réel (gènes *aflD* et *aflP* pour AFB1 et gènes *fum1* et *fum8* pour FB1). Aucun des gènes étudiés n'a été réprimé de manière significative chez *A. flavus* exposé aux 3 huiles essentielles. De plus, l'action des HEs sur la voie de biosynthèse des fumonisines est contrastée. Enfin, afin d'obtenir plus d'explications sur la façon dont les huiles essentielles limitent l'accumulation de mycotoxines, il serait judicieux d'étudier le transcriptome dans son ensemble par une approche expérimentale non ciblée telle que RNAseq sur plusieurs jours d'exposition du champignon au HEs.

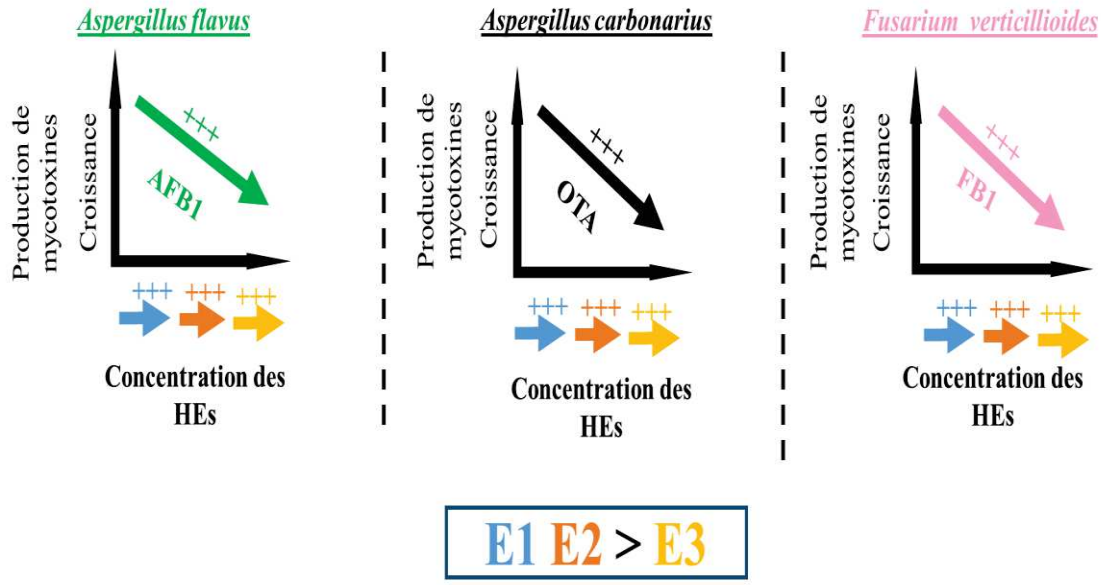


Figure 16 : Principaux résultats obtenus après la confrontation des souches mycotoxinogènes avec les huiles essentielles en méthode directe et en volatilisation.

En résumé, les résultats mettent en évidence l'effet des extraits de plantes étudiés (Antoferine® et trois huiles essentielles) sur la croissance et/ou la production de mycotoxines. Ces extraits apparaissent comme une solution prometteuse pour le biocontrôle du risque mycotoxique mais il est nécessaire d'élargir l'étude à d'autres souches fongiques mycotoxinogènes et de vérifier au champ les effets observés *in vitro*. Également, l'étude des mécanismes d'action devra être approfondie.

Perspectives

Perspectives

Suite aux résultats obtenus au cours de cette thèse, différentes perspectives peuvent être envisagées.

Il serait souhaitable de poursuivre les travaux en se concentrant sur l'étude des mécanismes d'actions de ces extraits naturels en investiguant les relations entre la structure des composés actifs des extraits des plantes et leurs activités biologiques (antifongique et/ou anti-mycotoxines).

Il serait envisageable d'étudier l'effet de ces extraits en :

- Ciblant d'autres **gènes** des voies de biosynthèse des **AFB1** et **FB1** afin d'avoir plus d'informations sur l'effet des extraits testés sur l'expression des gènes.
- Testant différentes **concentrations** de l'**Antoferine®** sur la détoxification des mycotoxines en utilisant également d'autres **paramètres abiotiques** pour identifier les conditions optimales.
- Identifiant les **composés de détoxification** et évaluer leur **toxicité**.
- Evaluant la capacité des **huiles essentielles** à **détoxifier** les mycotoxines.
- Identifiant les **composés volatils** des HEs responsables des effets inhibiteurs de la croissance et de la production de mycotoxines.
- Caractérisant **les constituants actifs** des extraits afin d'étudier d'éventuels **synergies ou antagonismes**.
- Etudiant les extraits naturels d'intérêt sous l'angle de **leur formulation et de leur stabilité** en vue d'une utilisation *in situ*.